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INHIBITION OF *NEISSERIA GONORRHOEAE* EPITHELIAL CELL INTERACTIONS BY VAGINAL *LACTOBACILLUS* SPECIES

presented by

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INHIBITION OF *NEISSERIA GONORRHOEAE* EPITHELIAL CELL INTERACTIONS BY VAGINAL *LACTOBACILLUS* SPECIES

By

Rachel R. Spurbeck

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ABSTRACT

INHIBITION OF *NEISSERIA GONORRHOEAE* EPITHELIAL CELL INTERACTIONS BY VAGINAL *LACTOBACILLUS* SPECIES

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Urogenital infections, including the sexually transmitted disease gonorrhea, are one of the main reasons women visit the doctor in the United States. One potential approach to the treatment or prevention of vaginally acquired infections is through the use of beneficial microbes known as probiotics. Most probiotic research, in both the gastrointestinal and vaginal tracts, has focused on species from the genus *Lactobacillus* since this genus is a member of the indigenous microbiota that is associated with health. The presence of *Lactobacillus* species in the vaginal tract has been correlated with a reduced risk of acquiring sexually transmitted infections upon exposure, and therefore has the potential for development as a vaginal probiotic. In this work *Lactobacillus jensenii*, one of the most common species of lactobacilli in the healthy human vaginal tract, was found to inhibit the interaction of *Neisseria gonorrhoeae* with epithelial cells at the levels of adherence and invasion. This inhibition was not due to direct killing of the pathogen or co-aggregation, as has been suggested in the case of uropathogens. Further characterization of the inhibition of adherence mediated by *L. jensenii* determined that the inhibition was independent of an epithelial cell response and due to *Lactobacillus* surface-associated proteins. By characterizing a cell-free preparation of released surface components from *L. jensenii*, it was shown that one of the surface associated inhibitory proteins was an enolase. The surface bound enolase inhibits gonococcal adherence to epithelial cells when produced

recombinantly in *E. coli* and purified as a C-terminal His_6-tagged protein, and retains enolase activity. Finally, it was determined that the substrate-binding site of the enzyme, but not the enolase activity, is necessary for inhibition of gonococcal adherence. The discovery of a protein from a commensal organism of the healthy human vaginal tract that inhibits gonococcal adherence is significant, and may lead to the production of new therapeutics to treat and prevent the sexually transmitted disease, gonorrhea, as well as other vaginally acquired infections.

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KEY TO SYMBOLS OR ABBREVIATIONS

BV ..... Bacterial Vaginosis

CFU ..... Colony Forming Unit

CO_2 ..... Carbon Dioxide

DMEM ..... Dulbecco's Modified Eagle Medium

ECM ..... Extracellular Matrix

FCS ..... Fetal Calf Serum

F^-+P_i ..... Fluoride And Inorganic Phosphate

Fn ..... Fibronectin

GAPDH ..... Glyceraldehyde-3-Phosphate Dehydrogenase

GC supp II ..... 12.4 μM $Fe(NO_3)_3$

GCV ..... GC Agar + Vancomycin

His_6-Eno ..... Epitope Tagged Enolase Protein

HIV ..... Human Immunodeficiency Virus

H_2O_2 ..... Hydrogen Peroxide

HRP ..... Horseradish Peroxidase

LEE ..... Locus of Enterocyte Effacement

LOS ..... Lipooligosaccharide

LTA ..... Lipoteichoic Acid

MeOH ..... Methanol

MRS ..... de Man, Rogosa, And Sharpe Medium

Opa ..... Opacity Protein

P^+ ..... Piliated

P^- ..... Non-pPiliated

PBS ..... Phosphate Buffered Saline

PEP ..... Phosphoenolpyruvate

2PGE 2-Phosphoglycerate

p.i. ..... Post Infection

PID ..... Pelvic Inflammatory Disease

PCR ..... Polymerase Chain Reaction

PAGE ..... Polyacrylimide Gel Electrophoresis

ProK ..... Proteinase K

RSC ..... Released Surface Components

RT ..... Reverse Transcription

semiQ ..... Semi-Quantitative

Slps ..... Surface Layer Proteins

STI ..... Sexually Transmitted Infection

TMB ..... 3,3',5,5'-Tetramethylbenzidine

WT ..... Wild Type

CHAPTER ONE

Spurbeck R. R., and Arvidson, C.G. (2010). Lactobacilli at the front line of defense against vaginal infections.

ABSTRACT

Probiotics, organisms that provide a health benefit to the host, are being studied for the treatment or prevention of infectious diseases. One of the most rapidly developing areas in which probiotics are being studied is in the management of vaginally acquired pathogens. Most of the work has centered on determining which bacterial species from the genus *Lactobacillus* have potential as probiotics. Several species have been found to produce compounds that inhibit the growth of pathogenic microorganisms, such as hydrogen peroxide, lactic acid, and bacteriocins. Other lactobacilli demonstrate an ability to reduce the adherence of pathogens to vaginal epithelial cells, including the bacterial vaginosis associated pathogen, *Gardnerella vaginalis*. However, more research is required to demonstrate what mechanism(s) these organisms use to defend the vaginal tract against infection *in vivo*. This review discusses the known mechanisms by which vaginal lactobacilli inhibit pathogen colonization *in vitro*. Other mechanisms that still need to be investigated to conclude what factors of the indigenous microbiota are necessary and sufficient for prevention of diseases of the female reproductive tract are also discussed.

INTRODUCTION

Infections of the female reproductive tract, such as vaginitis, pelvic inflammatory disease (PID), vulvovaginal candidiasis, bacterial vaginosis (BV), and sexually transmitted infections (STIs), are among the most common reasons women visit the doctor in the United States. In 2008, there were over 1.5 million cases in the United States of the three reportable STIs [chlamydia (1,201,664), gonorrhea (332,899), and syphilis (13,637)] (27). Since asymptomatic STIs often go undetected, the reported numbers are probably far lower than the actual incidence of these diseases.

If an STI is left untreated, serious and potentially irreversible complications can result. It is estimated that 30-40% of all female infertility is due to damage caused by an STI that ascended to the fallopian tubes. In fact, 10-40% of chlamydial infections develop into PID, which is an infection of the uterus, fallopian tubes, ovaries, and the surrounding tissues (118). PID affects approximately 1,000,000 women per year in the United States and it is estimated that 10% of those women will become sterile as a result (184). Due to the mild or absent symptoms of PID, over two thirds of all PID cases go undiagnosed until fertility problems arise (184). Women who have had at least one episode of PID are 6-10 times more likely to have an ectopic pregnancy, with an estimated 40-50% of all ectopic pregnancies being attributable to PID (184), demonstrating the need for better control of vaginal infections.

While reproductive problems are a major concern when women are infected, another critical issue is that the transmission of the human immunodeficiency virus

(HIV) is facilitated by the presence of an STI. In 2008, 56,300 new HIV infections were reported in the United States, with 31% of these new infections being attributed to high risk heterosexual contact (28). Gonorrhea and chlamydia, the two most common bacterial STIs in the United States, are known to increase the shedding of HIV leading to an increased transmission to sexual partners (64, 123). Vulvovaginal candidiasis has also been correlated with increased shedding of HIV (64).

BV is a dysbiosis of the vaginal microbiota that is characterized by a shift from a bacterial community dominated by the genus *Lactobacillus* to an overgrowth of anaerobic Gram-negative rods and *Gardnerella vaginalis* (99). This shift in the bacterial community is also correlated with an increase in the vaginal pH (100). BV is associated with an increased risk of acquiring HIV, gonorrhea, Chlamydia and vulvovaginal candidiasis (99, 187). Therefore, identification of a method to prevent dysbiosis of the vaginal microbiota could reduce the spread of STIs, HIV, and decrease the incidence of vulvovaginal candidiasis. This would lessen the amount of resources needed for treatment of vaginal infections and improve the quality of life for women at risk for these diseases.

INDIGENOUS MICROBIOTA

Since Elie Metchnikoff discovered microbes in Bulgarian yogurt that seemed to prolong life in 1907, microorganisms have been shown to impact human health (71). This discovery led to research of the interactions between commensal organisms and the host. Several bacterial species indigenous to the mucosal surfaces of the human body were consequently found to be beneficial to the health of the host. These good microbes, now called probiotics, are defined as "live microorganisms which

when administered in adequate amounts confer a health benefit on the host (47). While the majority of probiotic research has been conducted on the gastrointestinal microbiota, relatively little has been done on inhabitants of the vaginal tract (58).

The vaginal microbiota is a diverse community comprised of at least 40 bacterial genera (146). In healthy women of childbearing age, eight distinct supergroups of microbial species have been associated with normal indigenous microbiota, with *Lactobacillus* being the predominant genus in five (195). Lactobacilli are present at concentrations of 10^7-10^8 CFU/ml of vaginal fluid (135), and the four most prevalent species are *L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii* as determined by culture independent techniques (129, 172, 194-195).

Lactobacilli dominate the vaginal microbiota due to several factors. First, they are facultative anaerobic or anaerobic, allowing them to survive in the oxygen-limited environment of the vaginal tract. Second, in the post-menarchal/pre-menopausal vaginal tract, there is an accumulation of glycogen in the vaginal epithelium due to the elevated level of estrogen; lactobacilli use this glycogen as a substrate for growth (122, 189). Through the fermentation of glycogen, lactobacilli produce lactic acid which contributes directly to the low pH of the vaginal tract (pH 4-4.5) (21, 122, 172).

To maintain their presence in the vaginal tract it is thought that lactobacilli must adhere to the epithelium otherwise they would be removed by the continual flow of vaginal fluid through the mucosal layer. However, most *Lactobacillus* isolates adhere poorly to vaginal epithelial cells (5). In a study examining the composition of the microbial community in vaginal biopsy specimens isolated from healthy premenopausal women, the microbiota was not tightly adhered to the epithelium.

Instead, the lactobacilli and other microbes associated with the healthy state were found to be primarily in the vaginal fluid and mucosal layers (162). However, in biopsy specimens obtained from women with BV, *Gardnerella vaginalis* produced tightly adherent biofilms on the surface of the epithelium (162). This demonstrates that in a state of perturbation the community is more closely associated with the vaginal epithelium than when in a state of health. However, most research focuses on lactobacilli that are highly adherent to vaginal epithelial cells *in vitro* (20, 102), thereby overlooking some species that may have potential as probiotics. Two of the most common vaginal *Lactobacillus* species, *L. crispatus* and *L. jensenii*, were determined to most consistently colonize the vaginal tract in a study over a period of eight months (172), and therefore may be the best candidates for sustainable probiotics. In addition, epidemiological evidence shows that women colonized with either *L. crispatus* or *L. jensenii* in the vaginal tract and/or rectum were less likely to have BV than women colonized with other species of lactobacilli (10), supporting the role of these organisms in protecting the vaginal tract from infection.

Epidemiological evidence suggests that women colonized with high levels of vaginal lactobacilli are less susceptible to STIs such as gonorrhea and chlamydia (187). Lactobacilli and their products have been shown to affect the growth of *Neisseria gonorrhoeae in vitro* (143). In one study, H_2O_2 was found to be the major inhibiting factor (156); however, gonococci produce catalase which breaks down H_2O_2 into H_2O and O_2. The activity of this enzyme increases when gonococci are co-cultured with lactobacilli at a neutral pH *in vitro* (193). However, at low pH (~4-5) the production of catalase decreased, and the inhibition of gonococcal growth was

observed, therefore, H_2O_2 and low pH may be necessary for an effect on the growth of *N. gonorrhoeae* (193). To further complicate the situation, gonococci utilize lactate as a carbon source (152), therefore, whether lactobacilli can kill gonococci *in vivo* has yet to be determined.

Several studies have shown that lactobacilli can inhibit steps in the infection process including adherence of pathogens associated with urinary tract infections, vulvovaginal candidiasis, and BV (13-15, 102, 119-120, 157, 170, 191). Furthermore, lactobacilli were found to be able to disperse biofilms associated with BV (145). All of these observations support the premise that lactobacilli act as protective agents against vaginal infections; however, few studies have been conducted *in vivo* to verify the biological relevance of these results.

MECHANISMS BY WHICH VAGINAL LACTOBACILLI INHIBIT PATHOGEN COLONIZATION

Lactobacilli are thought to utilize several mechanisms to inhibit pathogen colonization of the vaginal tract. The most studied mechanism is direct killing of the pathogen by compounds secreted by lactobacilli, such as lactic acid, H_2O_2, and bacteriocins. Only the production of lactic acid has been demonstrated *in vivo*, and therefore, is the lone mechanism that has known biological relevance at this time. Other inhibitory mechanisms studied using *in vitro* systems, such as cell culture models, are co-aggregation between the pathogen and the lactobacilli, and competition for adherence to host cell receptors. However, both co-aggregation and competition for host receptors have not been demonstrated as of yet *in vivo*, therefore, the biological relevance of these phenomena is unknown. To increase the applicability of

probiotics for the treatment and prevention of vaginal infections, it is necessary for these proposed mechanisms to be examined *in vivo*.

Direct killing

Lactobacilli produce several compounds that can inhibit the growth of urogenital pathogens including lactic acid, H_2O_2, and bacteriocins (158). Lactic acid produced by lactobacilli contributes to the low pH (~ 4-6) of the vaginal tract, inhibiting the growth of bacteria that are not acid tolerant (21). For example, a vaginal isolate, *L. acidophilus* CRL 1259, was found to kill uropathogenic *Escherichia coli* under conditions that favored lactic acid production (67). Likewise, supernatants from *L. salivarius* subspecies *salivarius* CRL 1259 cultures were shown to inhibit the growth of *E. coli*, *Klebsiella sp.*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *G. vaginalis* due to the low pH caused by lactic acid production (116) In another study, it was determined that the amount of inhibition by a given species of *Lactobacillus* correlated with the amount of lactic acid produced (105). Therefore, acidification of the vaginal tract by lactobacilli is a major factor in protecting against pathogen colonization.

H_2O_2 generated by lactobacilli is another compound that can kill incoming pathogens (135, 157, 172, 191). It has been determined that 96-98% of vaginal *Lactobacillus* isolates from healthy women are H_2O_2-producers (46, 134), whereas only 6-23% of lactobacilli isolated from women with infections associated with preterm delivery, produced H_2O_2 (77). Pre-term delivery is thought to be induced by infections of the vaginal tract that ascend to the uterus causing inflammation of the

lower uterine pole (188). BV is associated with preterm delivery, as early acquisition during pregnancy correlates with a higher risk of premature birth (188). The presence of H_2O_2-producing *Lactobacillus* strains, including *L. jensenii*, *L. crispatus*, and *L. gasseri*, is associated with a reduced risk of bacterial vaginosis, pre-term birth, and chorioamnionitis, which is one of the main causes of fetal and neonatal death (188). H_2O_2-producing lactobacilli are also correlated with a reduced risk of acquiring sexually transmitted pathogens, such as *N. gonorrhoeae, C. trachomatis,* and HIV (17, 80, 147, 187). Furthermore, the hydrogen peroxide produced by several *Lactobacillus* species has been shown to inhibit the growth of G. *vaginalis*, *S. aureus*, and *N. gonorrhoeae* (114-115, 156, 167). Therefore, the focus has been on H_2O_2-producing *Lactobacillus* strains for the development of probiotics. However, H_2O_2-production by lactobacilli has not been measured *in vivo*; therefore, the biological relevance of H_2O_2 in the prevention of vaginal diseases has yet to be determined.

Bacteriocin production is also involved in direct killing of microorganisms by lactobacilli. Bacteriocins are bactericidal proteins produced by bacteria to reduce competition for shared resources (72). Several bacteriocin-like compounds are produced by lactobacilli that affect the growth of bacteria from the same genus as well as a variety of Gram-positive and Gram-negative pathogens (72, 94, 164). A bacteriocin from the culture supernatant of *L. salivarius* subspecies *salivarius* CRL 1259 was able to inhibit the growth of *Enterococcus faecalis* and *N. gonorrhoeae* demonstrating that it can inhibit both Gram-positive and Gram-negative bacteria (116). Another bacteriocin isolated from *L. fermentum* L23 was also fungicidal (127).

Some of the most prevalent vaginal *Lactobacillus* species (*L. gasseri, L. crispatus*, and *L. jensenii*) have been found to produce bacteriocins (68, 73). The bacterocins produced by these *Lactobacillus* strains had microbicidal activity against the vaginal pathogens *G. vaginalis*, *C. albicans*, and *E. coli* (68). Even so, a study of 45 human vaginal isolates of the species *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. plantarum*, determined that none produced bacteriocins (100). This suggests that bacteriocins are not generally produced by vaginal lactobacilli, and may be a minor factor in the reduced risk of transmission observed in epidemiological studies. Nevertheless, the microbicidal activity associated with bacteriocin producing strains suggests there is potential for their use as a probiotic treatment of BV or vulvovaginal candidiasis, although further research is necessary to demonstrate that bacteriocins are effective *in vivo*.

Co-aggregation

Lactobacilli could also protect the vaginal tract from infection by aggregation with the incoming pathogen, thereby titrating the pathogen away from the surface of the host epithelium. This would allow it to be removed more effectively by the vaginal fluid and/or killed by antimicrobial products secreted by the lactobacilli (102). Some vaginal lactobacilli, including *L. gasseri, L. jensenii*, and *L. crispatus*, have been shown to co-aggregate with uropathogenic *E. coli* (UPEC). However, there were differences in the amount of co-aggregation seen, with the most prevalent lactobacilli co-aggregating less efficiently than rarer isolates (45, 79). *Lactobacillus* sp. HV 142, a producer of aggregation promoting factor (APF), co-aggregated with a wider spectrum of UPEC strains than most lactobacilli tested. This may suggest that APF expressing

strains could be more effective against UPEC (79). Lactobacilli have also been shown to co-aggregate with *G. vaginalis* and *C. albicans* (102). Again, co-aggregation varied from no aggregation to complete aggregation, differing between strains as well as between species (102). The two most highly aggregative, *L. salivarius* FV2 and *L. gasseri* MB 335, were also strong H_2O_2 producers and had significant bactericidal effects against *G. vaginalis*, seeming to support the hypothesis that aggregation promotes killing of the pathogen. However, these strains were not fungicidal, therefore, the ability to co-aggregate with a pathogen does not correlate directly with the ability to kill the pathogen (102). Taken together, co-aggregation may enhance the inhibition of pathogen colonization, but is not a universal mechanism utilized by vaginal lactobacilli. This mechanism has yet to be tested *in vivo*, which would determine if co-aggregating lactobacilli should be examined further for use as probiotics.

Direct competition for host cell receptors

Another mechanism by which lactobacilli could protect the human vaginal tract is through competition with pathogenic microbes for host cell receptors. If a pathogen adheres to epithelial cells via the same receptors already occupied by the indigenous lactobacilli, then its adherence will be inhibited by exclusion. Several *Lactobacillus* species isolated from the vaginal tract have been shown to exclude *G. vaginalis, P. bivia C. albicans, S. aureus, S. agalactiae, E. coli, K. pneumoniae*, and *Pseudomonas aeruginosa* from adhering to host epithelial cells (13-15, 68, 119-120, 191). Furthermore, if lactobacilli have a higher affinity than the pathogenic bacteria for the host receptors, they could displace already adherent pathogens and potentially

be used as a treatment for vaginal infections. Some lactobacilli have been shown to displace *G. vaginalis* and *C. albicans*, suggesting that lactobacilli could be used to treat BV or vulvovaginal candidiasis (20, 102). However, it is not yet clear what factors lactobacilli use to exclude or displace pathogens from the host epithelium. Therefore, more in depth studies will be necessary to identify such factors and determine their mechanism of action.

A cell-free preparation of lipoteichoic acid complexed with peptidoglycan (LTA-peptidoglycan) from a *Lactobacillus* strain was shown to exclude the uropathogens *E. coli*, *K. pneumoniae*, and *P. aeruginosa* from uroepithelial cells (30). However, the authors hypothesized that the exclusion of pathogen adherence was likely due to steric hindrance, rather than direct competition for receptors, as the bulky LTA-peptidoglycan complex could inhibit pathogens from reaching adjacent receptors (30). *L. paracasei* CRL 1289, a strain that can inhibit the growth of *S. aureus* by production of H_2O_2, also reduces pathogen adherence by competing directly for host cell receptors (191). Using a mouse model, *L. paracasei* CRL 1289 was shown to not only reduce the inflammatory response to *S. aureus* infection but also reduce the number of the pathogenic bacteria colonizing the vaginal tract when infected with a relatively low infectious dose. However, the lactobacilli were unable to protect the mice when the infectious dose was increased (192). Therefore, more work is needed to improve the probiotic strain before use as a preventative for *S. aureus* vaginal infections.

While research on the adherence factors of vaginal *Lactobacillus* isolates is rather limited, some have been characterized for intestinal lactobacilli. The one study

pertaining to vaginal lactobacilli suggests that *L. acidophilus* and *L. gasseri* use glycoproteins while *L. jensenii* utilize carbohydrates to adhere to vaginal epithelial cells (20). In the intestinal tract, it has been shown that lactobacilli utilize proteins (54, 56, 110, 138), carbohydrate components, and/or lipoteichoic acid as adhesins (1-2, 20, 31, 53-54, 56, 113). These adherence factors bind to several different substrates including colonic mucus, and extracellular matrix (ECM) proteins such as fibronectin, laminin, and collagen (3, 6, 8, 26, 59, 70, 89, 140, 151).

In *L. reuteri*, some of the proteinaceous adhesins have been defined to the molecular level, such as MapA, a mucus adhesion promoting protein, which binds to human intestinal epithelial cells (110). Also, a collagen binding protein is expressed by *L. reuteri* (3, 140), and was determined to be part of a biosurfactant preparation known to inhibit uropathogen adherence (55). Other lactobacillar adhesins involved in binding to ECM are enzymes normally found in the cytoplasm such as enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (7, 26, 144). These enzymes are surface associated on several lactobacilli, and can be shed by changing the pH of the medium (8). However, the involvement of these adhesins in pathogen inhibition or competition for receptors has yet to be characterized.

Surface layer proteins (Slps) have also been implicated in the adherence of lactobacilli to epithelial cells, and may play a role in the interference with pathogen colonization. Slps are located outside of the bacterial cell wall and form a protective paracrystalline layer around the bacterium (63). Slps from the intestinal probiotic strain *L. heleveticus*, were shown to inhibit the binding of *E. coli* O157:H7 to intestinal epithelial cells (63), thus indicating that these proteins can affect the adherence of

pathogens. However, the role of Slps as inhibitors of pathogen adherence in the vaginal tract has not been demonstrated. Of the *Lactobacillus* species that dominate the microbiota of the vaginal tract, only *L. crispatus* produces a surface layer, and the Slp was determined to be a collagen binding protein (166). APF, the autoaggregation promoting factor expressed in some strains of *L. gasseri*, has also been suggested to be a Slp (178). However, *L. jensenii*, and some strains of *L. gasseri*, do not produce surface layers (18, 129); therefore, the role of Slps in inhibition of vaginal pathogen adherence is yet to be determined.

Given that the adherence mechanisms of vaginal lactobacilli are still relatively uncharacterized, identification of the adhesins used by these organisms would greatly advance the field of probiotics by allowing more sustainable probiotics to be developed. Since several vaginal lactobacilli are thought to directly compete with pathogens for receptors on epithelial cells, understanding the adherence mechanisms of lactobacilli, would enable predictions to be made as to which probiotic would exclude or displace specific pathogens.

POTENTIAL VAGINAL *LACTOBACILLUS* MECHANISMS OF PATHOGEN INHIBITION

Several mechanisms have proposed to inhibit pathogen colonization that have yet to be examined in the vaginal tract, such as biosurfactants, mucin production, increased barrier function, immunomodulation, and change in virulence gene expression. However, lactobacilli from the gastrointestinal tract have been shown to utilize some of these mechanisms. Since there may be niche specific mechanisms for pathogen

inhibition, the mechanisms that have been elucidated in the intestinal tract should be studied for relevance for the vaginal tract.

Biosurfactants

Biosurfactants are amphipathic molecules, which contain both hydrophobic and hydrophilic domains (139). These compounds, which can be protein, carbohydrate, or glycoproteins, are utilized by microbes for a variety of purposes, such as emulsification of hydrocarbons, quorum sensing, biofilm regulation, competition by antimicrobial activity, and regulation of attachment and detachment (139). A biosurfactant-producing organism can use the surfactant as an adhesin, allowing the bacteria to adsorb to the surface through hydrophobic or hydrophilic interactions. Upon detachment from the substrate, some bacteria leave behind the biosurfactant molecules, changing the chemistry of the surface. This alteration could interfere with the adherence of an incoming bacterial species reducing its colonization of that surface.

More than 15 strains of lactobacilli have been shown to produce biosurfactant activity. Some of the lactobacillar biosurfactants are secreted, whereas others are associated with the surface of the bacterium (137). While the effect of biosurfactants on vaginal pathogens is unknown, there is evidence that biosurfactants produced by vaginal lactobacilli effect uropathogen adherence. Biosurfactants produced from *Lactobacillus fermentum* B54 and *Lactobacillus acidophilus* RC-14 [also known as *L. fermentum* RC-14 and *Lactobacillus reuteri* RC-14], isolates from the healthy human vaginal tract, were tested for effects on the adherence of *E. faecalis* to glass surfaces. These were compared to the effect of a biosurfactant produced from *L. casei* subsp.

rhamnosus 36, isolated from a woman with a history of urogenital infections (177). The strains isolated from the healthy vaginal tract were able to reduce the initial deposition of *E. faecalis* onto the glass, whereas *L. casei* subsp. *rhamnosus* 36 did not significantly affect the adherence of *E. faecalis* to glass (177). In a similar study, the biosurfactant from *L. acidophilus* RC-14 (surlactin) was able to inhibit the adherence of *E. faecalis* to silicone rubber (176). The ability of surlactin to inhibit the adherence of the uropathogens *C. albicans*, *K. pneumoniae*, *Providencia stuartii*, *P. aeruginosa*, *E. coli*, and *Staphylococcus epidermidis* to silicone rubber has since been studied (175). Surlactin could inhibit the adherence of all of these pathogens to varying degrees. *C. albicans* was inhibited the least, with only a 12% reduction seen after 4 h incubation. *S. epidermidis* was the most affected, with an 82% inhibition observed within the same time period (175). Since both silicone rubber and glass can be used in catheters, the ability of *Lactobacillus*-produced biosurfactants to inhibit the adherence of uropathogens to these substrates could potentially be utilized to reduce the incidence of urinary tract infections in catheterized patients. Furthermore, surlactin inhibited *S. aureus* adherence to surgical implants and abscess formation by 89% in a rat model (51), and biosurfactants produced by other lactobacilli have also been shown to inhibit *S. aureus* biofilm formation (183). Therefore, biosurfactants produced by lactobacilli could play a significant role in reducing nosocomial infections, and need to be examined further to determine the involvement of these molecules in the inhibition of vaginal pathogen colonization.

Mucin production

The vaginal tract is lined by a thick mucosal layer that serves as a physical barrier against microbial colonization. Mucin, the main component of mucus, is a glycoprotein which forms a gel-like network over the epithelial cells (141). This matrix is difficult for incoming microorganisms to penetrate. In the gastrointestinal tract, adherent lactobacilli have been shown to increase the production of mucins by the epithelium by increasing the expression of mucin genes (4, 76, 92-93, 103). This increase in mucin production in response to *Lactobacillus* inhibits the adherence of both enterohemorrhagic and enteropathogenic *E. coli* strains to the epithelial cells, thereby reducing pathogen colonization (76, 93). While studies on the effect of lactobacilli on the production of mucin in the vaginal tract have yet to be conducted, it has been demonstrated that adherence of *C. albicans* is reduced when cervical mucins are present (36). Thus it is conceivable that increased mucin production could reduce vaginal pathogen adherence.

Increasing barrier function

Some vaginal pathogens, such as *N. gonorrhoeae*, are known to invade or translocate through the epithelium to the subepithelial space (104, 108). Therefore, a mechanism to reduce bacterial invasion or increase the barrier function of the epithelium could reduce the progression of vaginal infections. Lactobacilli are known to inhibit damage caused by *E. coli* O157:H7 to the barrier function of intestinal epithelial cells, reducing the severity of the disease (62). During an infection, *E. coli* O157:H7 reduces intercellular adhesion between intestinal cells and transmembrane electrical resistance, however, when pre-treated with *Lactobacillus rhamnosus* GG,

these effects were prevented (62). Furthermore, several *Lactobacillus* species have been found to protect tight junction proteins from damage caused by enteroinvasive *E. coli* (133), *Shigella dysenteriae* (111), and *E. coli* O157:H7 (62, 132). It is still to be determined if lactobacilli can protect the vaginal tract against similar damage.

Immunomodulation

Another mechanism probiotic lactobacilli utilize in the gastrointestinal tract to prevent infectious diseases is immunomodulation of the host (49, 117, 171, 180). Lactobacilli can increase the production of secretory immunoglobulin A (sIgA) in the colon and ileum, which traps pathogens in the mucosal layer, preventing its colonization (97-98, 180). Intestinal lactobacilli have also been shown to increase $CD4^+$ and $CD8^+$ cells in the gastrointestinal tract, which in turn, leads to an increase in active phagocytes and could reduce the amount of exogenous bacteria available to colonize the host, thus reducing the risk of pathogen colonization (98, 171, 180). Furthermore, intestinal lactobacilli can induce anti-inflammatory cytokines and reduce the expression of pro-inflammatory cytokines (49, 76, 180), thus decreasing the damage caused by the host inflammatory response to infection.

Interference with pathogen gene expression

Several pathogenic bacteria are known to modify virulence gene expression upon entering the host (39, 82, 106). One mechanism utilized is autoinducer-mediated quorum sensing, where the pathogens only express virulence genes when a specific concentration, or quorum, of the bacteria has been reached (23, 82, 106). It has recently been shown that probiotic organisms can inhibit pathogen colonization of the host by interfering with quorum sensing, thereby inhibiting the expression of virulence

factors necessary for persistent colonization. For instance, *L. reuteri* RC-14, by an unknown mechanism, disrupts *S. aureus* quorum sensing causing a decrease in the expression of staphylococcal superantigen-like protein and the accessory gene regulator (agr) locus (85). Similarly, *L. acidophilus* La-5 has been shown to secrete a molecule that interferes with the quorum sensing regulation of the locus of enterocyte effacement (LEE) in *E. coli* O157:H7 by reducing the concentration of extracellular autoinducer-2 (106). The reduced expression of the LEE locus correlated with decreased colonization of intestinal epithelial cells, demonstrating that lactobacilli can inhibit pathogen colonization by reducing virulence gene expression (106). This area of research is relatively new and could lead to exciting new therapeutics such as autoinducer-2 inhibitors to reduce bacterial pathogen virulence.

CONCLUSION

Lactobacilli are the most prevalent microorganisms in the indigenous microbiota of the vaginal tract, and the presence of these organisms has been correlated with a reduced risk of vaginal infections. Therefore the use of these microbes as probiotics to treat and/or prevent vaginal infections has recently become an exciting topic of interest. Much has been done to examine which *Lactobacillus* species are effective in treating or preventing BV and vulvovaginal candidiasis. Comparatively little has been done to examine the effects of lactobacilli on the interactions of sexually transmitted pathogens with the vaginal epithelium. Since adherence is an essential first step in an infection of the vaginal tract, it is important to discern the effect lactobacilli may have on the two most common bacterial infections, gonorrhea and Chlamydia. To further the field of vaginal probiotics, future study

needs to include attention to mechanistic detail as well as differences in *Lactobacillus* strains. It is clear that vaginal lactobacilli utilize multiple factors to reduce competition with exogenous bacteria, and how these factors inhibit pathogens *in vivo* must be understood in order to develop effective probiotics. Once it is known what specific adhesins are used by each probiotic strain of lactobacilli, better predictions of the efficacy against particular pathogens can be deduced. Studies of vaginal probiotics should continue to include the mechanisms of action proposed for intestinal lactobacilli, as well as considering other possibilities. It is likely that some mechanisms for reduction of pathogen colonization are niche specific, necessitating further studies in systems specific to the female reproductive tract. *In vivo* experiments would then be required to determine if the effective mechanisms of inhibition seen *in vitro* can translate to clinical purposes.

SIGNIFICANCE OF THE CURRENT WORK

The aim of the current work was to determine the effects of vaginal *Lactobacillus* species on the interactions of *N. gonorrhoeae* with host epithelial cells. *N. gonorrhoeae* (gonococcus), an obligate human pathogen, is the causative agent of gonorrhea, and infects an estimated 62 million people per year worldwide (www.who.org). If left untreated, gonorrhea can lead to PID, disseminated gonococcemia, gonococcal arthritis, sterility or ectopic pregnancy. Asymptomatic infections are also very common, and it is estimated that 50% of gonorrheal infections are undiagnosed (27). Due to similarities with other sexually transmitted infectious agents, and since it is amenable to laboratory manipulation, *N. gonorrhoeae* can serve as a model for development of new treatment and prevention methods.

The pathogenesis of gonorrhea has been well characterized. In the initial phase of a gonococcal infection in a female host, *N. gonorrhoeae* adheres to receptors on epithelial cells of the genital tract, which include CD46 (42), complement 3 receptor (42, 44), lutropin receptor (153), glycolipids (126), fibronectin (41, 174), vitronectin (34), collagens I and IV(41), and several types of proteoglycans (109). Type IV pili are the primary adhesins (160), although several additional surface structures can mediate adherence, including lipooligosaccharide (LOS, (43)) porin (PI, (173)) and opacity proteins (Opa, PII, (95, 182)). The process of adherence is a crucial step in mounting a successful infection; if the pathogen cannot adhere, it will be washed away by the flow of vaginal fluid. Once *N. gonorrhoeae* has adhered to the epithelium, gonococci invade the host cells and transcytose to the subepithelial space, where they elicit the acute inflammatory response characteristic of gonorrhea. *N. gonorrhoeae*, as an incoming pathogen, resists some of the host's innate defenses by the production of IgA protease (88), catalase (193), sialyation of lipooligosaccharide (96), phase and antigenic variation of surface structures (33, 84), and blockage of IgG (65). However, the effect of lactobacilli on the interactions of gonococci with the host has not been elucidated.

In 2007, *N. gonorrhoeae* was classified as a "superbug" due to the acquisition of resistance to most antibiotics used to treat gonorrhea. Recent gonococcal isolates have been shown to be resistant to penicillins, tetracyclines, spectinomycin, and most recently, fluoroquinolones. This excessive and rapidly developing antibiotic resistance of gonorrhea has led the CDC to recommend only 3rd generation cephalosporins to treat all gonococcal infections (www.cdc.gov). However, it is only a matter of time

before resistance to these drugs arise. Therefore, it is imperative that new therapies for the treatment and prevention of gonorrhea be explored. One potential target for therapeutics is at the critical initial step of adherence to host tissues, and methods to prevent adherence of gonococci to the host epithelium would be effective in preventing an infection before it begins. As previously stated, altered states in the microbiota can increase the risk for transmission of STIs, thus it is likely that the indigenous microbes of the vagina play a role in preventing pathogen colonization. Study of the innate mechanisms of the vaginal microbiota have potential to lead to the development of new therapeutics to treat and/or prevent vaginal infections. In this dissertation, I ascertained the effect of *Lactobacillus jensenii* on gonococcal adherence to and invasion of epithelial cells, and then systematically determined what *L. jensenii* factors inhibit pathogen adherence, starting with the mechanisms discussed above.

CHAPTER TWO

Spurbeck R.R. and C.G. Arvidson. (2008) Inhibition of *Neisseria gonorrhoeae* Epithelial Cell Interactions by Vaginal *Lactobacillus* species. Infect. and Immun. **76(7)**: 3124-30.

ABSTRACT

High levels of *Lactobacillus*, the dominant genus of the healthy human vaginal microbiota, have been epidemiologically linked to a reduced risk of infection following exposure to the sexually transmitted pathogen, *Neisseria gonorrhoeae*. In this work, a cell culture model of gonococcal infection was adapted to examine the effects of lactobacilli on gonococcal interactions with endometrial epithelial cells *in vitro*. Pre-colonization of epithelial cells with *L. jensenii*, *L. gasseri* 33323, or *L. gasseri* 9857 reduced gonococcal adherence by nearly 50%. Lactobacilli also inhibited gonococcal invasion of epithelial cells by more than 60%, which was independent of the effect on adherence. Furthermore, lactobacilli were able to displace adherent gonococci from epithelial cells, suggesting that these organisms have potential as a post-exposure prophylactic. Thus, vaginal lactobacilli have the ability to inhibit gonococci at two key steps of an infection, which might have a significant effect in determining whether the gonococcus will be able to successfully establish an infection following exposure *in vivo*.

INTRODUCTION

Neisseria gonorrhoeae (gonococcus), the causative agent of gonorrhea, is an obligate human pathogen that infects an estimated 62 million people per year worldwide (www.who.org). Gonorrhea is treatable with antibiotics; however, the disease is still prevalent due in part to the high frequency of asymptomatic and subclinical infections in women, providing a significant reservoir for transmission. Untreated gonococcal infections ascend the reproductive tract in up to 45% of infected women and can lead to disseminated infection, pelvic inflammatory disease, ectopic pregnancy, or sterility. Thus, gonococcal infections place a significant health burden on our society.

In the initial phase of a gonococcal infection in a female host, *N. gonorrhoeae* adheres to receptors on epithelial cells of the genital tract. Type IV pili are the primary adhesins (160), although several additional surface structures can also mediate adherence, including lipooligosaccharide [LOS; (43)], porin (173), and opacity proteins [(Opa, PII; (95, 182)]. The process of adherence is a crucial step in mounting a successful infection; if the pathogen cannot adhere, it could be swept away from the epithelial cell surface by the flow of vaginal fluid. While the flow of vaginal fluid (on average 1.55 g/8 h) is not as rapid as that of urine, if the pathogen is non-adherent, it will eventually be cleared or killed by the antimicrobial agents present in the vaginal mucus (52, 189).

The indigenous microbiota plays an important role in protecting the host from colonization by incoming pathogens. *Lactobacillus* is the predominant genus in the vaginal (195) and endocervical microbial communities (189) present at concentrations

of 10^7-10^8 CFU/mL of vaginal fluid in healthy post-menarchal/pre-menopausal women (135). *L. jensenii* and *L. gasseri* are two of the most common species present as determined by culture-independent techniques (129, 195). Communities dominated by lactobacilli seemed to be more resilient and less susceptible to altered microbial states such as bacterial vaginosis (BV (195)). BV, which is in part defined as a lack of lactobacilli, has been correlated with an increased risk of sexually transmitted infections (STIs (187)). This suggests that women who have large numbers of vaginal lactobacilli are less susceptible to STIs, such as gonorrhea and chlamydia, than women with BV.

While the epidemiological evidence suggests that the presence of lactobacilli is correlated with a reduced susceptibility to gonococcal infections, there has been no direct evidence linking pre-colonization by lactobacilli and effects on *N. gonorrhoeae* at any step in the infection process in cell culture or *in vivo*. Two studies have shown that lactobacilli and their products, such as hydrogen peroxide, can affect the growth of *N. gonorrhoeae in vitro* (143, 156); however, these experiments were conducted in the absence of host cells, so any inhibitory effects lactobacilli might have on gonococcal interactions with epithelial cells would not have been observed. *In vivo*, interactions between lactobacilli and gonococci are likely different due to the presence of the host. In this work, we hypothesized that lactobacilli affect gonococcal adherence to and invasion of epithelial cells, and have adapted a tissue culture model of gonococcal infection to investigate the effect of lactobacilli on these interactions.

MATERIALS and METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* MS11 [P^+ Tr (148)] was grown at 37°C in a humidified 5% CO_2 environment (+ CO_2) on GC agar (Accumedia, Lansing, MI) with supplements (75) and VCNT inhibitor (Becton, Dickinson, and Company, Sparks, MD). *L. jensenii* [ATCC 25258, $H_2O_2^+$ (156), human vaginal origin] and *L. gasseri* [ATCC 33323, $H_2O_2^+$ (156), human unknown tissue of origin, and ATCC 9857, human vaginal origin] were grown at 37°C + CO_2 on MRS agar (Difco, Sparks, MD).

Hydrogen peroxide production. All *Lactobacillus* strains were tested for hydrogen peroxide production utilizing the plate assay developed by Eschenbach *et al* (46) and modified by Felten *et al* (48). Briefly, MRS agar plates were made containing 0.25 mg/mL 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich, St. Louis, MO), and 0.01 mg/mL horseradish peroxidase (HRP; Sigma-Aldrich). Each strain was streaked for isolated colonies on MRS-TMB plates and on MRS-no TMB control plates that contained 0.01 mg/mL HRP and 225 μL EtOH (diluent for TMB). The plates were incubated for 48 hours at 37°C + CO_2. Hydrogen peroxide produced by the lactobacilli reacts with HRP to produce O_2, which then reacts with TMB to form a dark blue precipitate. Colonies of hydrogen peroxide-producing strains are stained dark blue.

The production of hydrogen peroxide by *L. jensenii*, *L. gasseri* 33323, *L. gasseri* 9857, and *N. gonorrhoeae* individually and in co-culture was also measured in

cell culture medium using an assay described previously (40, 130-131) with some modifications. Briefly, lactobacilli and gonococci were swabbed from fresh plate cultures into Dulbecco's Modified Eagle Medium without phenol red (DMEM, Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS, Invitrogen), GC supplement II [12.4 μM $Fe(NO_3)_3$], and 110 mM sodium pyruvate. Bacterial concentrations were determined spectrophotometerically, and diluted to 10^7 CFU/mL for lactobacilli and 10^6 CFU/mL for gonococci. Serial dilutions were plated on appropriate selective media (lactobacilli, MRS; *N. gonorrhoeae*, GCV) to quantify inocula. Bacterial cultures were incubated at 37°C + CO_2 for 3 h, and were then centrifuged for 10 min at 3428.5 x g (3000 RPM) and filtered through a 0.22 μm (pore-size) membrane. 0.75 mL of peroxide assay buffer [5.0 mM K_2HPO_4, 1.0 mM KH_2PO_4, 140 mM NaCl, 0.5 mM glucose, with phenol red (final concentration 0.46 mM) and horseradish peroxidase (final concentration 0.046 U/mL) added immediately prior to the assay] was placed into a borosilicate glass tube to which 0.25 mL of the sample supernatants were added. The samples were incubated for 30 min at 37°C, and then NaOH (final concentration 0.004 N) was added to stop the reaction. The absorbance at 610 nm was determined spectrophotometrically, and the amount of hydrogen peroxide produced under each condition was determined by comparing to a H_2O_2 standard curve generated in the same assay.

Cell culture. The human endometrial epithelial cell line Hec-1-B (ATCC HTB-113) was grown in DMEM (Invitrogen) supplemented with 5% FCS at 37°C + CO_2. Cell

culture assays were carried out in 24-well cell culture plates with Hec-1-B cells grown to 50-90% confluency. Adherence and invasion assays were as described previously (12, 39) with modifications as follows.

Adherence assay 1. Lactobacilli were swabbed from fresh plate cultures into DMEM (without phenol red) supplemented with 5% FCS, GC supplement II, and 110 mM sodium pyruvate (DMEM/5% FCS/GC supp II). Bacterial concentrations were determined spectrophotometerically, and diluted in DMEM/5% FCS/GC supp II to a concentration of 4×10^7 CFU/mL. Serial dilutions of this inoculum were plated on MRS agar. Hec-1-B cells were pre-colonized with 250 μL of the lactobacilli inoculum for a multiplicity of infection (MOI) of 100, and the infected epithelial cells were incubated at 37°C + CO_2 for 3 h. The epithelial cells were then washed 5 times with phosphate buffered saline (PBS) to remove non-adherent lactobacilli. A mock infection was conducted as a control in which the epithelial cells were incubated with 250 μL DMEM/5% FCS/GC supp II for 3 h and then washed 5 times with PBS prior to the introduction of gonococci. Gonococci were swabbed from fresh plate cultures into DMEM/5% FCS/GC supp II. Bacterial concentrations were determined spectrophotometerically, and diluted in this medium to a concentration of 4×10^6 CFU/mL. Serial dilutions of this inoculum were plated on GCV agar. Hec-1-B cells pre-colonized with adherent lactobacilli, along with the control, were then infected with 250 μL of the *N. gonorrhoeae* inoculum (MOI of 10) and the epithelial cells were incubated at 37°C + CO_2 for 3 h. Following incubation, the medium was removed and the infected epithelial cells were washed five times with sterile PBS to remove non-

adherent bacteria. The epithelial cells and cell-associated bacteria were lifted with 1 mL PBS containing 5 mM EDTA (PBS/EDTA). Serial dilutions were plated on GCV agar to determine the cell-associated CFU. The CFU of gonococci adherent to epithelial cells pre-colonized with lactobacilli was then normalized to the cell-associated CFU from the control, which lacked lactobacilli.

Adherence assay 2. *L. jensenii* was swabbed from fresh culture plates into DMEM/5% FCS/GC supp II. Bacterial concentrations were determined and diluted in this medium to 4 x 10^6 CFU/mL and 4 x 10^7 CFU/mL. Serial dilutions of each inoculum were plated on MRS agar. One row of 6 wells containing 10^5 Hec-1-B cells was pre-colonized with 250 μL of the 4 x 10^6 CFU/mL lactobacilli inoculum (MOI of 10) and a second row of wells containing 10^5 Hec-1-B cells was pre-colonized with 250 μL of the 4 x 10^7 CFU/mL lactobacilli inoculum (MOI of 100). A third row was used as a mock infection in which the media on the epithelial cells was replaced with 250 μL fresh DMEM/5% FCS/GC supp II. The infected epithelial cells were then incubated for 1 h at 37°C + CO_2. Gonococci were swabbed from fresh plate cultures into DMEM/5% FCS/GC supp II. Bacterial concentrations were determined spectrophotometerically, and diluted to 4 x 10^6 CFU/mL. Serial dilutions of this inoculum were plated on GCV agar. Hec-1-B cells pre-colonized with adherent and non-adherent lactobacilli, along with the control containing no lactobacilli, were then infected with 250 μL of the *N. gonorrhoeae* inoculum (MOI of 10) and the infected epithelial cells were incubated at 37°C + CO_2 for 3 h. Following incubation, the

infected Hec-1-B cells were split into two sets, one set was to calculate the total CFU of bacteria, and the other set was to calculate the cell-associated (adherent) CFU. For the first set, the supernatant (0.5 mL) from each well was placed in a sterile tube. The Hec-1-B cells and cell-associated bacteria were then lifted with 500 μL of PBS/EDTA and added to the sterile tube containing the unattached bacteria. Serial dilutions were then plated on appropriate selective media to determine the total CFU/well. For the second set, the supernatant was removed and the epithelial cells were washed five times with sterile PBS. The epithelial cells and cell-associated bacteria were then lifted with 1 mL PBS/EDTA and placed in a sterile tube. Serial dilutions were plated on selective media to obtain the cell associated (adherent) CFU/well. The adherence frequency was calculated by dividing the cell-associated CFU/well by the total CFU/well. The adherence frequencies were then normalized to the control, which contained no lactobacilli.

Invasion assays. Pre-colonization of the Hec-1-B cells with *L. jensenii* was conducted as described above for adherence assay 2, with the lactobacilli inoculum at a concentration of 4×10^7 CFU/mL. After incubation for 1h, 250 μL of *N. gonorrhoeae* (4×10^6 CFU/mL) was added to the Hec-1-B cells pre-colonized with lactobacilli and incubated for 7 h. Following incubation, wells were divided into three sets. For the first set (total CFU), the supernatant (0.5 mL) was removed and placed in a sterile tube. The Hec-1-B cells and cell-associated bacteria were lifted with 500 μL PBS/EDTA, and this suspension was added to the tube of supernatant. Serial dilutions were plated on selective media to determine the total CFU/well. For the second set (cell-associated CFU), the epithelial cells were washed 5 times with PBS to remove

non-adherent bacteria, and the Hec-1-B cells with adherent bacteria were then lifted with 1 mL PBS/EDTA. Serial dilutions were plated on selective media to determine the cell-associated CFU/well. The third set of wells (intracellular CFU) was washed five times with PBS to remove non-adherent bacteria, treated with 75 mg/L gentamicin (Gm) in DMEM and then incubated at 37°C + CO_2 for 1 h to kill extracellular bacteria. The Hec-1-B cells (with intracellular bacteria) were then washed 5 times with PBS and lifted with 1 mL PBS/EDTA. Serial dilutions were plated on selective media to determine the Gm^R CFU/well. The invasion frequency is defined as the ratio of Gm^R CFU/well to cell associated CFU/well.

Microscopy. Hec-1-B cells were seeded into 6 well cell culture plates containing sterilized glass coverslips (25 mm, Fisher Scientific). Once the epithelial cells reached confluence, an adherence assay as described above for adherence assay 2 was carried out with some modifications. Briefly, at 3 h post infection (p.i.), non-adherent bacteria in the supernatant were removed and placed on glass microscope slides and Gram stained. The bacteria were then visualized by light microscopy (magnification 100X) and photographed using a digital still camera (Cyber-shot DSC-P50, SONY). The epithelial cells with adherent bacteria were washed five times with PBS and then fixed with 100% methanol for five minutes. The coverslips were then removed from the well, placed on microscope slides, and Gram stained. The bacteria adherent to the Hec-1-B cells were visualized by light microscopy (magnification 100X) and photographed using a digital still camera. The number of adherent lactobacilli was determined for thirty consecutive Hec-1-B cells in each replicate. Three replicate

experiments, carried out in duplicate were conducted and the adherent bacterial counts were then averaged.

Pre-conditioned medium. Hec-1-B cells were infected with *L. jensenii* at an MOI of 10 or 100, and incubated for 1 h at 37°C + CO_2. The supernatant from the infected epithelial cells was removed, centrifuged at 16,000 x g (14,000 RPM) for 30 sec to pellet the bacteria, and then filtered through a 0.22 μm membrane. These pre-conditioned media were then inoculated with 10^6 CFU gonococci and used to infect fresh Hec-1-B cells (MOI 10). The infected Hec-1-B cells were incubated at 37°C + CO_2 and at 3 h p.i., the epithelial cells with adherent bacteria were washed, lifted, and plated as described above for adherence assays.

Statistical analysis. All data was analyzed by unpaired student t-test. *P*-values of less than 0.05 were considered statistically significant, and are indicated by a * in each of the graphs.

RESULTS

Conditions for *N. gonorrhoeae* and *Lactobacillus* co-culture. *L. jensenii* and *L. gasseri* are two of the most prevalent *Lactobacillus* species recovered from the vaginal tract of healthy women (9, 22, 25, 194), and therefore were chosen for this study. Previous research showed that *L. jensenii* 25258 and *L. gasseri* 33323 had the potential to kill or inhibit the growth of *N. gonorrhoeae in vitro* (156). Thus, it was necessary to identify conditions in which both species could grow in order to study the effects on gonococcal interactions with epithelial cells. Each bacterial strain was grown separately in DMEM/5% FCS/GC supp II and dilutions were plated at intervals to determine viable CFU/mL. Next, *N. gonorrhoeae* (starting at 10^6 CFU/mL) was co-cultured with *L. jensenii*, *L. gasseri* 33323, or *L. gasseri* 9857, at a ratio of 1:10 (gonococci: lactobacilli) and plated for viable CFU at intervals to determine the effect of co-culture on growth of each bacterial strain. As shown in Figure 2.1, the growth of *N. gonorrhoeae* in the presence of *L. jensenii* was similar to that observed when grown separately. Similar results were observed when *N. gonorrhoeae* was grown in the presence of *L. gasseri* (data not shown). Each *Lactobacillus* strain also survived similarly in the presence and absence of gonococci (data not shown). These results establish that gonococci and lactobacilli survive and grow in co-culture under these conditions.

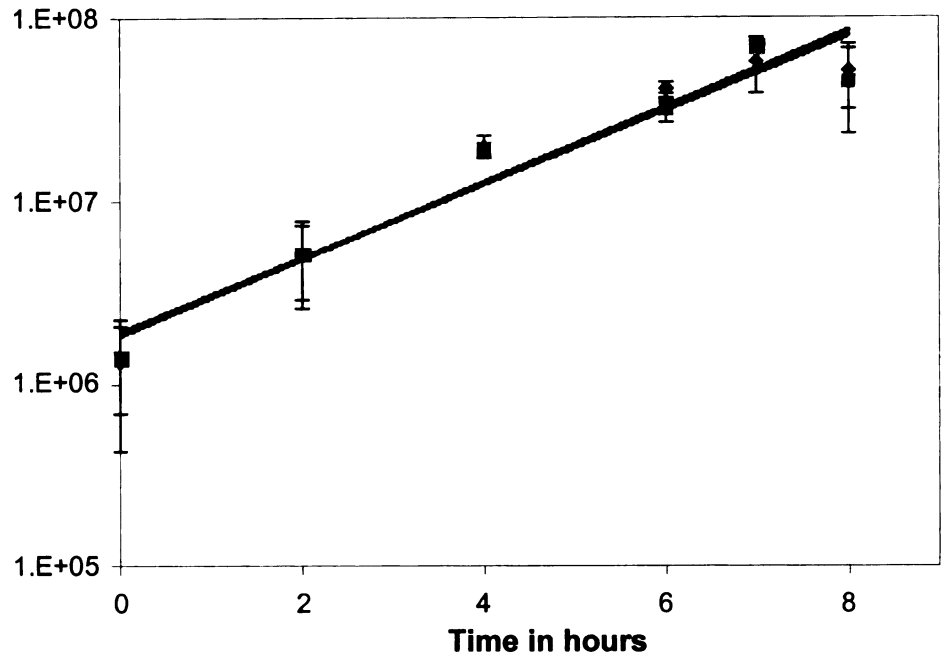

Figure 2.1 Growth of *N. gonorrhoeae* in the presence or absence of *L. jensenii*. *N. gonorrhoeae* (10^6 CFU/mL inoculum) was grown in the (□) presence or (◆) absence of *L. jensenii* (10^7 CFU/mL inoculum). Serial dilutions were plated on selective media at 2 h intervals to quantify CFU. Data are the averages of three independent experiments. Error bars indicate standard deviations.

Hydrogen peroxide production in cell culture medium. Two of the strains used in this work, *L. jensenii* (ATCC 25258) and *L. gasseri* 33323, were previously reported to kill *N. gonorrhoeae* in an agar overlay assay due to the production of hydrogen peroxide (61, 156). Under the conditions of our assays, the lactobacilli did not inhibit the growth of gonococci (Figure 2.1). Therefore, the *Lactobacillus* strains used in our study were tested for hydrogen peroxide production by an MRS-TMB plate assay (46, 48). Using this method, in which hydrogen peroxide production is visualized by the production of a dark blue precipitate, *L. jensenii* and *L. gasseri* 33323 were confirmed to be hydrogen peroxide producers (solid dark blue), while *L. gasseri* 9857 produced

modest levels of hydrogen peroxide (blue after a prolonged incubation). Next, hydrogen peroxide production by lactobacilli grown in our cell culture conditions in both the presence and absence of gonococci was examined. When incubated for 3 h in DMEM/5% FCS/GC supp II, *L. jensenii*, *L. gasseri* 33323, *L. gasseri* 9857, and *N. gonorrhoeae* all produced undetectable levels of hydrogen peroxide (less than 0.1 mM). When incubated in co-culture with *N. gonorrhoeae* in DMEM/5% FCS/GC supp II for 3 h, *L. jensenii*, *L. gasseri* 33323, and *L. gasseri* 9857 again produced undetectable levels of hydrogen peroxide. The lack of detectable levels of hydrogen peroxide is consistent with our observations that when co-cultured in DMEM/5% FCS/GC supp II lactobacilli do not kill gonococci, and with the findings of other researchers that at neutral pH, hydrogen peroxide producing lactobacilli do not inhibit the growth of gonococci (193).

Adherence of *N. gonorrhoeae* to epithelial cells pre-colonized with lactobacilli. Epidemiological evidence suggests there is a reduced risk of developing an infection following exposure to an STI, such as gonorrhea, when lactobacilli are present in large numbers (187). Therefore, the effect of pre-colonization of epithelial cells with lactobacilli on the adherence of *N. gonorrhoeae* was examined. Hec-1-B cells, a human endometrial epithelial cell line, were infected with lactobacilli at an MOI of 100, or DMEM/5% FCS/GC supp II (control). At 3 h p.i., non-adherent lactobacilli were removed by washing. Epithelial cells pre-colonized with lactobacilli and the control epithelial cells were then infected with gonococci at an MOI of 10. At 3 h p.i., the Hec-1-B cells were again washed to remove non-adherent bacteria, lifted, and plated on selective media. The adherence of each *Lactobacillus* strain to the epithelial

cells after exposure to *N. gonorrhoeae* was as follows: *L. jensenii* – 0.73%, *L. gasseri* 33323 – 0.61%, and *L. gasseri* 9857 – 0.93% of the inoculum. The average CFU/well of *N. gonorrhoeae* adherent to Hec-1-B cells pre-colonized with each *Lactobacillus* strain was compared to the average CFU/well of gonococci adherent in the control, and these results are shown in Figure 2.2. When Hec-1-B cells were pre-colonized with *L. jensenii,* gonococcal adherence dropped to 63.0 ± 21.9 % ($p = 0.043$) of that determined for the control. Pre-colonization with *L. gasseri* 33323 reduced gonococcal adherence to 56. 6 ± 13.8 % ($p = 0.003$), and pre-colonization with *L. gasseri* 9857 reduced gonococcal adherence to 63.0 ± 24.6 % ($p = 0.059$). Thus, in the presence of two of the adherent strains of *Lactobacillus* tested, the adherence of *N. gonorrhoeae* was significantly decreased as compared to the control.

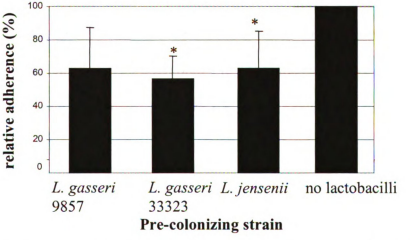

Figure 2.2. Adherence of *N. gonorrhoeae* to Hec-1-B cells pre-colonized with lactobacilli. Epithelial cells were incubated with lactobacilli for 3 h. Non-adherent lactobacilli were removed prior to the addition of gonococci, and the Hec-1-B cells were incubated an additional 3 h. Relative adherence is the percentage of adherent gonococci in wells containing lactobacilli divided by the percentage of adherent gonococci in the control. The data are averages of three independent experiments performed in duplicate. Error bars indicate standard deviations. * statistically significant.

Since the human vaginal tract contains lactobacilli in the fluid and mucosal layer that are non-adherent to the epithelia, and because adherence is a dynamic process with bacteria attaching and then detaching from the host cells (162), we next developed an approach to examine the adherence of *N. gonorrhoeae* in a system more closely mimicking this natural state. In the experiments described above, non-adherent lactobacilli were washed off prior to the addition of gonococci, leaving fewer than 10^5 CFU of lactobacilli adherent to epithelial cells in the well. It has been reported that there are 10^7-10^8 CFU/mL lactobacilli of vaginal fluid of healthy pre-menopausal women (135). Therefore, gonococcal adherence to Hec-1-B cells pre-colonized with lactobacilli was next examined without removing the non-adherent lactobacilli.

Hec-1-B cells were inoculated with *L. jensenii* at an MOI of 10 or 100, and incubated for 1 h. Hec-1-B cells were incubated with sterile DMEM/5% FCS/GC supp II for 1 h as a control. Gonococci were then added at an MOI of 10, and the infected epithelial cells were incubated an additional 3 h. The relative gonococcal adherence frequencies are shown graphically in Figure 2.3. At a 1:1 ratio of *N. gonorrhoeae* to *L. jensenii*, gonococcal adherence was reduced to 73.9 ± 22.1 % ($p = 0.11$) of the no lactobacilli control, whereas at the ratio 1:10 *N. gonorrhoeae* to *L. jensenii* the adherence of *N. gonorrhoeae* was reduced to 52.5 ± 14.2 % ($p = 0.004$). Thus, inhibition of gonococcal adherence increased with the number of lactobacilli present.

The adherent CFU per well of *L. jensenii* increased proportionally with the number of lactobacilli in the inoculum. At an MOI of 10, 4×10^4 CFU/ well *L. jensenii* were adherent to the epithelial cells (an adherence frequency of ~1% cell-

associated lactobacilli/total lactobacilli), while at an MOI of 100, 3 x 10^5 CFU/ well *L. jensenii* were adherent (an adherence frequency of ~1%). When the first adherence assay (Figure 2.2) and the second adherence assay (Figure 2.3) are compared, the CFU of adherent lactobacilli per well inversely correlates with the gonococcal adherence frequency, suggesting that as the number of adherent lactobacilli increases, the number of adherent gonococci decreases correspondingly.

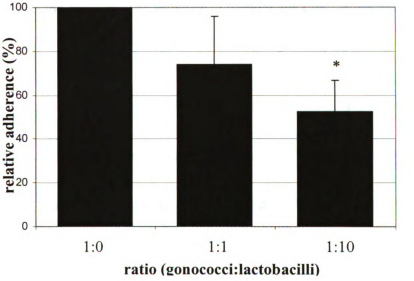

Figure 2.3. Adherence of *N. gonorrhoeae* to Hec-1-B cells in the presence of adherent and non-adherent lactobacilli. Relative adherence is the percentage of adherent gonococci in wells containing lactobacilli divided by the percentage of adherent gonococci in the control. The data are the averages of three independent experiments performed in triplicate. Ratios of gonococci to lactobacilli were: 1:0, 10^6 gonococci:no lactobacilli; 1:1, 10^6 gonococci:10^6 lactobacilli; 1:10, 10^6 gonococci:10^7 lactobacilli. At 3 h p.i. the CFU of *N. gonorrhoeae* doubled, and the total CFU of gonococci in the control was not significantly different from that of the *Lactobacillus* treated samples [*p*-values: 0.324 (1:1) and 0.536 (1:10)]. Error bars indicate standard deviations. Student's t-test *p*-values were 0.11 for 1:1 and 0.004 for 1:10. * statistically significant.

Gonococcal invasion of epithelial cells pre-colonized with lactobacilli. After adherence has been attained in a gonococcal infection, a subpopulation of these

bacteria invades the epithelial cells to transcytose to the subepithelial space. To determine whether lactobacilli could affect gonococcal invasion, a gentamicin (Gm) protection assay was used in which Hec-1-B cells were first pre-colonized for 1 h with *L. jensenii* at MOI 100 prior to the addition of gonococci (MOI 10). As a control, Hec-1-B cells were incubated with fresh DMEM/5% FCS/GC supp II for 1 h prior to inoculation with gonococci. The frequency of invasion was determined as Gm^R CFU/well per cell-associated CFU/well at 7 h p.i., thereby segregating the frequency of adherence from the frequency of invasion. To check for Gm^R gonococci that are not intracellular, 4 x 10^6 CFU/mL gonococci were incubated for 1 h with Gm at the same concentration used in the invasion assay and then plated dilutions from 10^1–10^5 for viable cell counts. There were less than one Gm^R gonococci in 4 x 10^6 CFU/mL, verifying that the gonococcal strain used in these experiments was not Gm^R. The results of the invasion experiments show that gonococcal invasion was reduced to 39.46 ± 10.47% ($p = 0.001$) in the presence of *L. jensenii* compared to invasion in its absence. Interestingly, at 7 h post (gonococcal) infection when invasion was measured, the adherence frequency for the gonococci in co-culture with *L. jensenii* was not significantly different from that of the control (data not shown). Thus, although gonococci eventually recover from the initial inhibition of adherence, these results indicate that lactobacilli affect both steps of gonococcal interactions with Hec-1-B cells.

Displacement of adherent gonococci by lactobacilli. To determine whether lactobacilli had the potential to affect gonococcal interactions with host epithelial cells post-exposure, we assessed the ability of lactobacilli to displace gonococci already adherent to epithelial cells. Hec-1-B cells were infected with *N. gonorrhoeae* at an MOI of 10, as for an adherence assay. At 3 h p.i., non-adherent gonococci were removed, and DMEM containing *L. jensenii*, *L. gasseri* 33323 (MOI of 100), or no bacteria (control) was added. Infected epithelial cells were incubated for an additional hour. Non-adherent bacteria were then removed and Hec-1-B cells with adherent bacteria were lifted and plated on selective media. After addition of *L. gasseri* 33323, the number of adherent gonococci was reduced by 28.7% ($p = 0.019$) as compared to the control (Figure 2.4). Displacement by *L. jensenii* was less consistent ($p = 0.174$), although treatment with either *Lactobacillus* species tended to reduce the number of adherent gonococci.

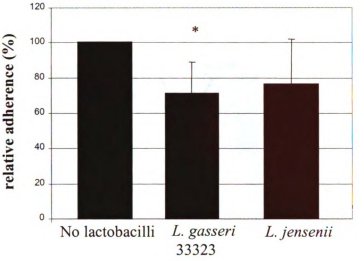

Figure 2.4. Displacement of *N. gonorrhoeae* adherent to Hec-1-B cells by lactobacilli. *N. gonorrhoeae* adherent to Hec-1-B cells after 3 h were then infected with *L. gasseri* 33323 or *L. jensenii* at an MOI of 100 for 1 h. Adherence data are presented as a percentage of adhered gonococci in the well divided by that of the control and are the averages of four or more experiments performed in triplicate. Error bars indicate standard deviations. Student's t-test *p*-values were 0.019 (*L. gasseri* 33323) and 0.174 (*L. jensenii*). *, statistically significant.

Spatial arrangement of lactobacilli and gonococci adherent to epithelial cells. To visualize the spatial arrangement of gonococci and lactobacilli on the epithelial cell monolayer during a co-infection, adherence assays were conducted in which the epithelial cells were adherent to a glass coverslip, allowing for microscopic analysis. Adherent lactobacilli were observed either individually or in short chains (1-3 bacilli), at an adherence frequency of 0.72 lactobacilli per epithelial cell, consistent with the adherence frequencies found using the plate count method. Gonococci were predominantly in microcolonies when adherent to epithelial cells in the presence or absence of lactobacilli, thus counting of individual gonococci adherent to Hec-1-B cells was not possible. In all fields observed, lactobacilli and gonococci were found

randomly dispersed on the epithelial cells and generally not in contact with each other (Figure 2.5A and B).

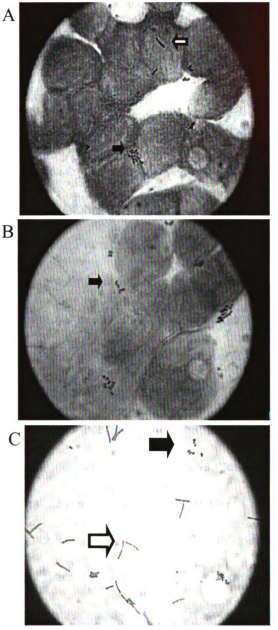

Figure 2.5. Gram stains from an adherence assay performed as for the experiment shown in Fig. 2.2 with Hec-1-B cells pre-colonized with 10^7 CFU *L. jensenii*. Panel A depicts gonococci and lactobacilli adherent to epithelial cells. Panel B depicts gonococci adherent to epithelial cells in the absence of lactobacilli. Panel C depicts the arrangement of gonococci and lactobacilli in the supernatant. In all panels the white arrow points to lactobacilli and the black arrow points to gonococci (Olympus model CHBS microscope, 100X magnification).

Lactobacilli and gonococci do not co-aggregate in co-culture. Previous reports have suggested that lactobacilli produce aggregation factors that could allow lactobacilli to aggregate with other bacterial species, thus sequestering pathogens and decreasing adherence (19, 79). To determine if co-aggregation of lactobacilli with gonococci could be the mechanism of inhibition observed in the adherence assays, the supernatants of three separate adherence assays (performed in duplicate) were Gram stained and visualized by light microscopy. In all fields observed, lactobacilli and gonococci never formed co-aggregates and a representative field of such an experiment is shown in Figure 2.5C.

***Lactobacillus* pre-conditioned medium does not inhibit gonococcal adherence.** Recently, *Lactobacillus acidophilus* was found to secrete a soluble compound that can reduce expression of virulence factors in *Escherichia coli* O157:H7 necessary for colonization of intestinal epithelial cells (106). To examine the possibility that lactobacilli secrete a soluble compound that inhibits gonococcal adherence to epithelial cells, gonococcal adherence assays were performed using *Lactobacillus* pre-conditioned media. *L. jensenii* at an MOI of 10 (PC-10) and an MOI of 100 (PC-100) were incubated in cell culture medium with Hec-1-B cells for 1 h and then removed by centrifugation and filtration. These media were then inoculated with gonococci at an MOI of 10 for an adherence assay using fresh Hec-1-B cells and compared to a control of gonococci inoculated in fresh media. The results of these experiments show that the gonococcal adherence frequency was not statistically different from that of the control in either PC-10 (p = 0.631), or PC-100 (p =0.884, Figure 2.6).

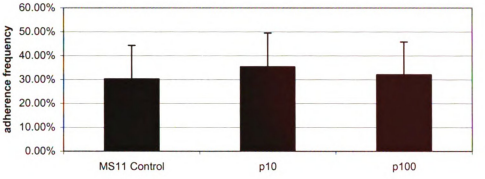

Figure 2.6. Adherence of gonococci in medium pre-conditioned with *L. jensenii* and Hec-1-B cells. Percent adherence is the ratio of adherent gonococci to the total number of gonococci for each condition. Number of lactobacilli used to condition the medium: Fresh medium; no lactobacilli, fresh DMEM/5% FCS/GC supp II; P-10, 10^6 CFU lactobacilli:10^5 Hec-1-B cells; P-100, 10^7 CFU lactobacilli:10^5 Hec-1-B cells. Data are the average of four independent experiments performed in triplicate. Error bars indicate the standard deviation. P-10: $p = 0.631$, and P-100: $p = 0.884$.

DISCUSSION

To successfully infect the female genital tract, gonococci must first adhere to the epithelia to avoid clearance by the continual flow of vaginal fluid. A reduction in the ability to adhere would significantly affect the outcome of an infection following exposure. Although lactobacilli have been implicated in protecting the female vaginal tract from exogenous pathogens, research on the direct effect of lactobacilli on STIs has been lacking. Therefore, we developed a co-culture model of gonococcal infection that includes lactobacilli to examine their effect on gonococcal interactions with epithelial cells.

In our *in vitro* model of gonococcal infection, pre-colonization with lactobacilli reduced gonococcal adherence to epithelial cells by 40-50% (Figure 2.2 and 2.3). This inhibition of adherence was dependent on the ratio of gonococci to adherent lactobacilli, with the inhibition becoming more pronounced in the presence of increased numbers of lactobacilli (Figure 2.3). In a gonococcal infection, following adherence to epithelial cells, gonococci will invade these epithelial cells and transcytose to the subepithelial space. Our results show that gonococcal invasion of epithelial cells pre-colonized with *L. jensenii* was decreased by 60%. This inhibition of invasion was distinct from the inhibition of adherence, demonstrating that even the gonococci that overcome the inhibition of adherence are negatively affected in invasiveness by the presence of lactobacilli. The inhibition of adherence, along with the inhibition of invasion, suggests that colonization with lactobacilli could reduce the risk for infection by *N. gonorrhoeae* by potentially decreasing the amount of

gonococci that adhere and invade the mucosal epithelia, thus providing a greater opportunity for the innate host defenses to prevent the establishment of an infection.

Due to the nature of *N. gonorrhoeae* as an obligate human pathogen, testing the biological relevance of the 50% reduction in gonococcal adherence caused by lactobacilli cannot be tested *in vivo*. However, semen samples from males infected with gonorrhea on average contain 7×10^6 CFU per ejaculate (60), similar to the gonococcal inoculum used in these studies (6×10^6 CFU/well). Cervical aspirates of females with gonorrhea have been reported to contain 10^4-10^6 CFU/mL (91, 190). In our studies the average CFU of gonococci adherent to epithelial cells in the controls falls within this range (1×10^6 CFU/well), again lending support to the relevance of our experimental conditions. Thus, the inhibition of gonococcal adherence to and invasion of epithelial cells pre-colonized with lactobacilli may explain the epidemiological observations in which women with bacterial vaginosis (reduced numbers of lactobacilli) have an increased susceptibility for gonorrhea following exposure (187).

Lactobacilli are now being promoted as probiotic bacteria that have broad health benefits. Lactobacilli have the ability to displace adherent pathogens associated with urinary tract infections (137), as well as *Gardnerella vaginalis* biofilms associated with BV (145), suggesting potential as a treatment for these diseases. While it is unlikely that lactobacilli alone could be used as a treatment for gonorrhea, it is conceivable that lactobacilli could reduce the likelihood of gonococci establishing an infection when directly administered to the vaginal tract shortly after exposure. Reid *et*

al. (137) suggests a displacement of 20-50% would be clinically relevant for pathogens infecting the vaginal tract. Our results indicate that gonococcal displacement by *L. gasseri* 33323 is within this range at 28.7% (Fig 2.4). Thus, the ability of lactobacilli to displace adherent gonococci in a cell culture model of infection suggests that lactobacilli have potential as a post-exposure prophylactic for gonococcal infection.

In this study, *L. jensenii* and *L. gasseri* strains were chosen because these species are two of the most prevalent species recovered from healthy women and had been previously studied for effects on *N. gonorrhoeae* in the absence of epithelial cells (9, 22, 25, 194). Each strain inhibited the adherence of *N. gonorrhoeae* to epithelial cells, but adhered poorly to epithelial cells (average adherence frequency ~1%). When epithelial cells are pre-colonized lactobacilli at an MOI of 100, there is on average one adherent *Lactobacillus* per epithelial cell. Thus, it is unlikely that the mechanism for exclusion of gonococci is direct competition for receptors on the epithelial cells. However, the number of adherent lactobacilli is inversely correlated with the gonococcal adherence frequency: as the number of adherent lactobacilli increases the gonococcal adherence frequency decreases. This suggests that it is the adherent lactobacilli that play an important role in the observed inhibition of gonococcal adherence. Our results also show that the mechanism of inhibition of adherence is not due to co-aggregation of lactobacilli with gonococci (Figure 2.5C), growth inhibition (Figure 2.1), or the secretion of a stable soluble compound into the medium by either the epithelial cells or lactobacilli before inoculation with gonococci (Figure 2.6). Further study will be necessary to identify the mechanisms of inhibition, which will be

fundamental in the development of *Lactobacillus*-based treatments or preventatives for gonococcal infection.

In summary, we have adapted a tissue culture model of gonococcal infection to include a major constituent of the human vaginal tract, lactobacilli. This approach will be essential in elucidating the role of the indigenous microbiota in the early, and therefore preventable steps of a gonococcal infection. The observation that lactobacilli inhibit gonococcal adherence and invasion, and could even displace adherent gonococci is intriguing; however, the mechanisms by which these events occur are not yet clear. While co-aggregation, the secretion of soluble inhibitory molecules, and growth inhibition can be ruled out as possible mechanisms, there are still several hypotheses to test. Possible mechanisms might include a contact inducible change in the epithelial cells due to the adherence of lactobacilli that would reduce susceptibility to gonococci or the secretion of a short-lived inhibitory factor produced by lactobacilli directly in response to incoming gonococci. This model will be indispensable in future studies to examine the molecular basis for the observed inhibition of gonococcal interactions with epithelial cells.

CHAPTER THREE

Spurbeck R.R. and C.G. Arvidson. (2010) *Lactobacillus jensenii* Surface Associated Proteins Inhibit *Neisseria gonorrhoeae* Adherence to Epithelial Cells. Infect. and Immun. Epub: April 12, 2010.

ABSTRACT

High numbers of lactobacilli in the vaginal tract have been correlated with a decreased risk of infection by the sexually transmitted pathogen, *Neisseria gonorrhoeae*. We have previously shown that *Lactobacillus jensenii*, one of the most prevalent microorganisms in the healthy human vaginal tract, can inhibit gonococcal adherence to epithelial cells in culture. Here we examined the role of the epithelial cells and the components of *L. jensenii* involved in the inhibition of gonococcal adherence. *L. jensenii* inhibited the adherence of gonococci to glutaraldehyde-fixed epithelial cells similarly to live epithelial cells, suggesting that the epithelial cells do not need to be metabolically active for this inhibition to occur. In addition, methanol-fixed *L. jensenii* inhibited gonococcal adherence to live epithelial cells, indicating *L. jensenii* uses a constitutive component to inhibit gonococcal interactions with epithelial cells. Proteinase K treatment of the methanol-fixed lactobacilli abolished the inhibitory effect, suggesting that the inhibitory component contains protein. Released surface components (RSC) isolated from *L. jensenii* were found to contain at least two inhibitory components, both of which are protease-sensitive. Using anion exchange and size exclusion chromatography an inhibitory protein was isolated, which has significant similarity to the enzyme enolase. A recombinant His_6-tagged version of this protein was subsequently produced and shown to inhibit gonococcal adherence to epithelial cells in a dose-dependent manner.

INTRODUCTION

Neisseria gonorrhoeae (gonococcus) is an obligate human pathogen that causes the sexually-transmitted infection, gonorrhea. Gonorrhea is one of the most commonly reported infectious diseases, second only to chlamydia, with 336,742 cases reported in 2008 in the United States according to the Centers for Disease Control and Prevention. Gonorrhea is readily treated with antibiotics; however, *N. gonorrhoeae* has developed resistance to each antibiotic used historically, including penicillins, tetracyclines, spectinomycin, and most recently, fluoroquinolones. As of 2007, the CDC recommends only one class of antibiotics to treat all types of gonorrhea, the cephalosporins (29). Furthermore, women infected with gonorrhea often have asymptomatic infections, providing a significant reservoir for transmission. Undiagnosed, and therefore untreated gonococcal infections can lead to permanent damage of the female reproductive system resulting in infertility or ectopic pregnancy. Gonorrhea has also been reported to increase susceptibility to HIV infections (150). Therefore, a method to reduce the number of gonococcal infections that does not involve antibiotics would be beneficial for public health.

Adherence to the host epithelia is the first and most critical step in a gonococcal infection, thus, this step is a target of interest for the development of new therapeutics. By inhibiting gonococcal adherence to the epithelial cells, the pathogen could be washed away by the flow of vaginal or menstrual fluid, or killed by the antimicrobial agents found in the vaginal mucus, rendering the pathogen unable to establish an infection (189). One potential source for an anti-adherence treatment is probiotic bacteria. Probiotics are defined as "live microorganisms which, when administered in

adequate amounts, confer a health benefit on the host" (47). The bacterial genus most studied for its probiotic properties is *Lactobacillus*. Lactobacilli naturally inhabit both the gastrointestinal tract and the female reproductive tract of healthy humans. In the female reproductive tract, lactobacilli make up the majority of the indigenous vaginal and endocervical microbiota (189, 195). Lactobacilli have been shown to play a role in protecting women from infection by incoming pathogens including HIV (149), and epidemiological evidence suggests that women with high numbers of vaginal lactobacilli have a reduced susceptibility to gonorrhea and chlamydia following exposure (187). Clearly, lactobacilli are a key component of the human defense against colonization by sexually transmitted pathogens.

Lactobacilli utilize several mechanisms to prevent the colonization of incoming pathogens. These include direct killing of the organisms by hydrogen peroxide, bacteriocins, or lowering the pH of the vaginal tract to ~4 by the production of lactic acid (11, 14, 21). Lactobacilli can inhibit adherence by the production of biosurfactants (137, 177, 183), receptor competition (20, 30), or co-aggregation with the pathogen, allowing the pathogen to be swept away by the host's bodily fluids (19, 79). Lactobacilli can also inhibit pathogen colonization by causing the host cells to become more resistant to adherence (62, 76, 81, 133) or by suppressing the expression of virulence factors in the pathogen (106).

Lactobacillus jensenii, one of the most prevalent species of lactobacilli indigenous to the human vaginal tract, has been shown to inhibit gonococcal adherence to and invasion of epithelial cells (154). To date, the mechanism used by *L. jensenii* to inhibit gonococcal adherence is unknown. However, we have shown in our

previous work that *L. jensenii* does not directly inhibit the growth of gonococci or co-aggregate with the gonococci either in the medium or at the cell surface. Furthermore, the hydrogen peroxide produced by *L. jensenii* is also not effecting the gonococcal adherence to the epithelial cells (154). In the current work, we test the hypothesis that *L. jensenii* utilizes a surface component to inhibit gonococcal adherence and examine the processes and components of the epithelial cell, gonococci, and lactobacilli that play a role in this inhibition.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* MS11 (P^+, Tr) (148) and *N. gonorrhoeae* MS11-307 (Δ*pilE1::erm*; Δ*pilE2*, P^-, Tr, (107)) were grown at 37°C in a humidified 5% CO_2 environment (+ CO_2) on GC agar (Acumedia, Lansing, MI) with supplements (75) and VCNT inhibitor (GCV, Becton, Dickinson, and Company, Sparks, MD). *L. jensenii* (ATCC 25258, $H_2O_2^+$) was grown at 37°C + CO_2 on MRS agar (Becton, Dickinson). *Bacillus subtilis* strain RB 247 (169), was grown at 37°C on LB agar (Acumedia, Lansing, MI). *Escherichia coli* strains DH5α and BL21λDE3 was grown on LB agar at 37°C, with kanamycin at 100 mg/l (Kn^{100}) added for pET24a and derivatives.

Cell culture. The human endometrial epithelial cell line Hec-1-B (ATCC HTB-113) was grown in Dulbecco's Modified Eagle Medium (High glucose with L-glutamate, DMEM, Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS, Invitrogen) at 37°C + CO_2. Cell culture assays were carried out in 24-well cell culture plates with Hec-1-B cells grown to 50-90% confluency.

Glutaraldehyde fixation of Hec-1-B cells. Hec-1-B cells were incubated with 1 ml of glutaraldehyde (2.5% v/v) for 30 min at 37°C + CO_2. The glutaraldehyde was then removed and the fixed cells were washed 5 times with phosphate-buffered saline (PBS), immediately prior to use in adherence assays (153). Gonococcal adherence assays on glutaraldehyde fixed cells were carried out as shown below with the

exception that fixed cells were lifted with saponin (1% w/v in GCB) instead of PBS + 5 mM EDTA.

Methanol and proteinase K treatment of *L. jensenii*. An *L. jensenii* inoculum was divided into three 2 ml aliquots containing 4 x 10^7 CFU/ml. Aliquot 1 (live lactobacilli) was held at 37°C. Aliquots 2 and 3 were treated with an equal volume of ice cold methanol (MeOH) for 10 minutes, and then centrifuged at 14,000 x g for 3 min to pellet the bacteria. The supernatant was removed and replaced with 200 µl DMEM (Aliquot 2, MeOH-treated) or 160 µl DMEM and 40 µl proteinase K (ProK, 20 mg/ml, Aliquot 3, ProK-treated). All three aliquots were then incubated for 2 h at 37°C. Aliquots 2 and 3 were boiled for 3 min and then centrifuged at 14,000 x g for 3 min to pellet the bacteria. The supernatants were removed and the bacteria were resuspended in 2 ml fresh DMEM. Microscopic visualization of Gram stained samples confirmed that MeOH treatment and ProK treatment left the lactobacilli intact.

Adherence assays. Adherence assays were performed as previously described (154). Briefly, wells containing 10^5 Hec-1-B cells were inoculated with *L. jensenii* at an MOI of 100 (1 x 10^7 CFU/ml), *B. subtilis* at an MOI of 100 (1 x 10^7 CFU/ml), or fresh DMEM supplemented with 5% FCS, 12.4 µM $Fe(NO_3)_3$ (GC supplement II), and 110 mM sodium pyruvate (DMEM/5% FCS/GC supp II) as a mock infection. Following incubation at 37°C + CO_2 for 1 h, the epithelial cells were infected with *N. gonorrhoeae* at an MOI of 10 (1 x10^6 CFU/ml). Following 3 h of incubation at 37°C + CO_2, the infected Hec-1-B cells were divided into two sets: the first set was to

determine the total number of bacteria in the well, and the second set to determine the cell-associated (adherent) bacteria. For the first set, the supernatant from each well (0.5 ml) was placed in a sterile tube. The Hec-1-B cells, with the cell-associated bacteria, were then lifted with 0.5 ml of PBS + 5 mM EDTA (PBS/EDTA) and added to the supernatant. Serial dilutions were plated on appropriate selective media to quantify the total CFU/well. For the second set, the supernatant was removed and the epithelial cells were washed five times with sterile PBS. The epithelial cells with the cell-associated bacteria were then lifted with 1 ml PBS/EDTA and transferred to a sterile tube. Serial dilutions were plated on selective media to quantify the cell associated (adherent) CFU/well. The adherence frequency was calculated by dividing the cell-associated CFU/well by the total CFU/well. Where indicated, the adherence frequencies were normalized to the mock infection, which contained no lactobacilli.

Production of released surface components (RSC). A modification of the method of Reid *et al.* (137) was used to isolate released surface-associated proteins and biosurfactants from *L. jensenii*. Briefly, a 1 l culture of *L. jensenii* in MRS broth was grown to an A_{600} of 1.6-1.8, and the bacteria were harvested by centrifugation (10,000 x g for 10 min at 7°C). The bacterial cell pellet was washed twice with sterile H_2O. The cell pellet was then resuspended in 134 ml PBS and gently stirred for 2 h at room temperature. The *Lactobacillus* suspension was then centrifuged for 20 min at 3,000 x g and the supernatant was filtered (pore size 0.22 μm) to remove remaining bacteria. This cell-free preparation was concentrated to ~10 ml by Amicon ultraconcentration using a 10 kDa MWCO filter membrane. Protein concentration was determined using the Bradford method (Biorad, Richmond, CA). The products obtained were then

assayed for inhibition of gonococcal adherence to Hec-1-B cells by pretreating the epithelial cells for 3 h prior to infection with *N. gonorrhoeae*.

Fibronectin blocking assay. To assess the effect of fibronectin (Fn) on the inhibitory activity of the RSC, 0.42 mg/ml RSC was incubated with 50 μl of soluble Fn (0 – 10 μM) for 1 h at room temperature. This mixture was used to treat Hec-1-B cell monolayers (10^5 cells/well) for 3 h. The suspension was then removed and the cells washed once with PBS. An adherence assay with *N. gonorrhoeae* was carried out using these cells as described above.

Trypan blue exclusion assay. Wells containing 10^5 Hec-1-B cells were inoculated as for adherence assays above with either sterile DMEM, *L. jensenii*, *N. gonorrhoeae*, RSC, or a combination of *N. gonorrhoeae* and *L. jensenii* or *N. gonorrhoeae* and RSC. Following a 3 h incubation at 37°C + CO_2, the cells were washed 5 times with PBS, and then trypan blue (0.2% in PBS) was added. After 3 min, the trypan blue was removed and the cells washed once with PBS. The number of live and dead cells were counted visually and compared between all conditions.

Collapsed drop analysis. To assess biosurfactant activity, the RSC was assayed by a collapsed drop analysis as described by Walencka *et al.* (183). 2 μl of paraffin oil was allowed to equilibrate over night in the wells of 96-well microtiter plate lid. 5 μl of each sample was dropped onto the oil covered surface and observed for 1 h. The samples were scored as positive if the drop flattened (had biosurfactant activity) or negative if the drop remained beaded on the surface of the oil (no biosurfactant activity). Water was used as a negative control.

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Anion exchange column chromatography. After dialysis against H_2O, the RSC was brought to 50 mM Tris (pH 7.6) and separated by anion exchange column chromatography (Econo-Pac Q, Biorad) using a 0-1 M NaCl gradient in a buffer of 50 mM Tris, 5 mM EDTA. Column flow through and a 25 ml 0 M NaCl wash were collected before the gradient was run. Twenty-five 2 ml fractions were collected during the gradient and five 2 ml fractions were collected from a terminal 1 M NaCl wash. The protein in fractions was quantified using the Bradford method and samples were run on a 12% SDS-PAGE gel. The fractions were then dialyzed into PBS before being assayed for inhibitory activity against gonococcal adherence to Hec-1-B cells. Inhibitory fractions were also assayed for biosurfactant activity using the collapsed drop analysis described above.

Protein identification by tandem mass spectrometry. Proteins were separated on a 12% SDS PAGE gel and stained with 1% Coomassie Blue. The protein band of interest was excised from the gel and sent to the Michigan Proteome Consortium for analysis. Briefly, the sample was digested with trypsin, concentrated, and then spotted on a 192-well MALDI target and allowed to dry at room temperature. The sample was analyzed on a 4800 Proteomics Analyzer (TOF/TOF, Applied Biosystems) and the mass spectra were acquired in Reflector Positive Ion mode for a peptide range of 800-3500 kDa. MS spectra were summed from 2,000 laser shots from an Nd-YAG laser operating at 355 nm and 200 Hz. Internal calibration was performed using a minimum of 3 trypsin autolysis peaks. Database searching was performed using Applied Biosystems GPS Explorer v. 3.6, with Mascot v. 2.1. Spectra were subjected to a 7-point Gaussian smooth prior to peak picking. For the PMF search, a maximum of 65

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peaks were submitted with a S/N of 30, and a maximum peak density of 50 peaks per 200 Da. Data were searched against all *Lactobacillus* genome sequences in NCBI (www.ncbi.nlm.nih.gov/).

Cloning, expression and purification of recombinant *L. jensenii* enolase. The sequence of the enolase genes from *L. gasseri* 33323, *L. johnsonii* NC533, and all sequenced *L. jensenii* strains was compared using ClustalW2. Based on these sequences, primers were designed to amplify the putative enolase ORF from *L. jensenii* 25258. Initial attempts to amplify the entire open reading frame (ORF) were not successful. When conserved internal primers were used in combination with full length primers, it was determined that while the 5' end of the gene was highly conserved, the 3' end was not. To determine the sequence of the 3' end, a primer specific to the gene located 3' of the enolase gene in other lactobacilli (*secG*) was designed and used to PCR amplify the region. The DNA sequence of the product was determined and used to design a new primer for the 3' end of the enolase gene, EnoRev, which incorporated a *Not*I site. The 5' primer, EnoFwd, contained a *Nde*I site which included the ATG start codon. The ORF was PCR amplified, purified using a PCR purification kit (Qiagen, Germantown, MD) and then digested with *Not*I and *Nde*I (New England Biolabs, Ipswich, MA). The insert was ligated into similarly digested pET24a (Novagen, EMD4 Biosciences, San Diego, CA), and transformed into *E. coli* DH5α. Transformants were identified and the DNA sequence of the entire insert was determined. An isolate, pET24a-eno, was then transformed into *E. coli* BL21λDE3 for expression.

High level production of the His_6-enolase was achieved using the method described by Studier (159). Briefly, 50 mls of an overnight culture of BL21λDE3pET24a-eno grown in LB Kn^{100} at 37°C was used to inoculate 2 l of ZY-5052 autoinducing media supplemented with Kn^{100} and incubated overnight at 30°C with shaking at 250 rpm. The bacteria were harvested by centrifugation and resuspended in 50 mM NaH_2PO_4 pH 7.0, 300 mM NaCl. The cells were then lysed by shearing using a M-110P processor (Micro-fluidics Corp) set at 20k psi. The insoluble material was removed by centrifugation and the crude lysate was loaded onto a NiNTA resin column (BD Biosciences) that was equilibrated with 50 mM NaH_2PO_4 pH 7.0, 300 mM NaCl. The column was washed with 50 mM NaH_2PO_4 pH 7.0, 300 mM NaCl, 10 mM imidazole and then the bound protein was eluted using 50 mM NaH_2PO_4 pH 7.0, 300 mM NaCl, 250 mM imidazole. The protein was further purified by anion exchange chromatography as described above. Prior to use in cell culture assays, the protein was dialyzed against PBS and the protein concentration was determined using the Bradford method. Purified protein was then applied to epithelial cells at various concentrations for 3 h before infection with *N. gonorrhoeae*.

Statistical analysis. All data was analyzed by unpaired Student's t-test. A *p*-value below 0.05 was considered statistically significant and is indicated by asterisk (*) in the graphs.

RESULTS

***Lactobacillus* inhibition of gonococcal adherence to fixed epithelial cells.** In our experimental system, there are three players that could be involved in the *Lactobacillus* inhibition of gonococcal adherence to Hec-1-B cells: the epithelial cells, the lactobacilli, and the gonococci. Since intestinal *Lactobacillus* strains have been shown to induce epithelial cells to become more resistant to pathogen adherence (62, 76, 81, 133) we utilized glutaraldehyde-fixed Hec-1-B cells to determine the role of the epithelial cells in the inhibitory interaction. *L. jensenii* (MOI 100) was used to pre-colonize both glutaraldehyde-fixed and live epithelial cells for 1 h prior to gonococcal infection as described in the Materials and Methods. The cells were then lifted with saponin and plated onto selective media (MRS for lactobacilli, GCV for gonococci). On live epithelial cells, when *L. jensenii* is present, 8.9 ± 3.9% of the total gonococci present adhered, compared to 20.2 ± 8.0% adherence in the absence of lactobacilli. This is a significant reduction in the adherence of gonococci (Figure 3.1A, $p < 0.001$). This inhibition is specific to lactobacilli, as pre-treatment of the epithelial cells with *Bacillus subtilis* (MOI = 100) had no effect on the adherence frequency of gonococci when compared to the control (90.7 ± 13.5%, $p = 0.117$).

On fixed epithelial cells, gonococci adhere at a two-fold lower frequency than to live epithelial cells. Nonetheless, the presence of *L. jensenii* is able to reduce gonococcal adherence on fixed cells from 11.2 ± 2.8% to 4.4 ± 2.0% (Figure 3.1B, $p = 0.036$). The inhibition of gonococcal adherence to fixed epithelial cells was not significantly different from the level of inhibition seen for live epithelial cells ($p =$

0.433), suggesting that *Lactobacillus*-mediated inhibition of gonococcal adherence to epithelial cells does not require the epithelial cells to be metabolically active.

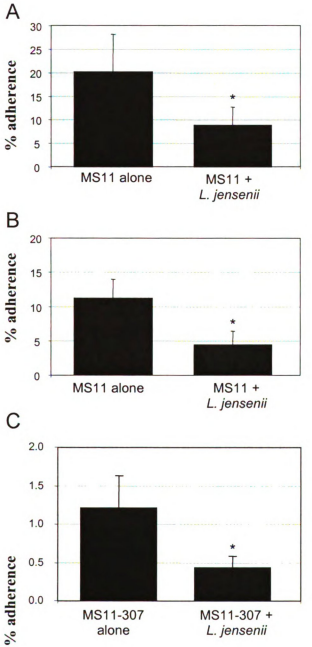

Figure 3.1. The effect of *L. jensenii* on gonococcal adherence. A. Adherence of *N. gonorrhoeae* to live Hec-1-B cells in the presence or absence of *L. jensenii*. The data are averages of 20 independent experiments performed in triplicate. Error bars indicate standard error. Student's t-test p-value < 0.001. B. Adherence of *N. gonorrhoeae* to fixed Hec-1-B cells in the presence or absence of *L. jensenii*. The data are averages of four independent experiments performed in triplicate. Error bars indicate standard error. Student's t-test p-value = 0.036. C. Adherence of non-piliated (MS11-307) gonococci in the presence and absence of *L. jensenii*. The data are averages of six independent experiments performed in triplicate. Error bars indicate standard error. Student's t-test p-value = 0.008.

Pilus-mediated gonococcal adherence is not targeted by *L. jensenii*. The type IV pilus is the primary adhesin for gonococci and has been shown to be necessary for infection (75, 161). We hypothesized that lactobacilli inhibit the adherence of *N. gonorrhoeae* by specifically targeting pilus-mediated adherence. The effect of *L. jensenii* on the non-piliated gonococcal strain MS11-307 (P^-) was examined to test this hypothesis. Non-piliated gonococci adhered to Hec-1-B cells at a frequency of 1.2 ± 0.4% (Figure 3.1C), which is significantly lower than that of the P^+ gonococcal strain (20.2 ± 8.0%, Figure 3.1A). Again, when the epithelial cells were pre-colonized with *L. jensenii*, the P^- gonococcal adherence frequency was reduced significantly to 0.43 ± 0.13% ($p = 0.008$, Figure 3.1C). After normalizing the data to the appropriate controls, gonococcal adherence frequencies were compared between the *L. jensenii* treated non-piliated and pilated gonococcal strains. No statistically significant difference was found ($p = 0.334$), which suggests that *L. jensenii* does not inhibit gonococcal adherence by specifically targeting type IV pilus-mediated adherence to epithelial cells.

Surface components of *L. jensenii* inhibit gonococcal adherence. Since we determined that the epithelial cells do not need to be metabolically active for *L. jensenii* to inhibit gonococcal adherence to host cells (Figure 3.1B), we next focused on determining the components of lactobacilli that are involved in this inhibition by comparing the effect of treating the lactobacilli with MeOH and/or ProK prior to infection with gonococci. As shown in Figure 2, MeOH-treated lactobacilli inhibited gonococcal adherence to epithelial cells 50.2 ± 25.9% ($p = 0.001$), similar to inhibition

observed with live lactobacilli 63.0 ± 14.9% ($p < 0.001$). This result suggests that the inhibitory factor is a surface component of *L. jensenii*. Additionally, ProK treatment abolished *Lactobacillus*-mediated inhibition of gonococcal adherence (96.9± 12.7%, $p = 0.533$). Taken together with the MeOH results, this suggests that a constitutive protein on the exposed surface of *L. jensenii* is involved in the inhibition of gonococcal adherence to epithelial cells.

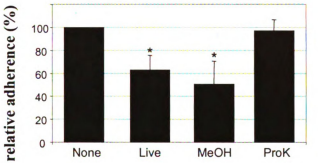

Figure 3.2. Adherence of *N. gonorrhoeae* in the presence of live, MeOH-fixed, and ProK-treated *L. jensenii*. Relative adherence is the percentage of adherent gonococci in the presence of lactobacilli divided by the percentage of adherent gonococci from the corresponding control. None, no lactobacilli; Live, untreated *L. jensenii*; MeOH, methanol-treated *L. jensenii*; ProK, proteinase K-treated *L. jensenii*. The data are averages of three or more independent experiments performed in triplicate. Error bars indicate standard error. Student's t-test *p*-values were <0.001 (live), 0.001 (MeOH) and 0.533 (ProK).

Released surface components (RSC) from *L. jensenii* inhibit gonococcal adherence to epithelial cells in a dose-dependent manner. *Lactobacillus* species have been shown to produce several compounds that can inhibit pathogen adherence, including hydrogen peroxide, bacteriocins, and biosurfactants (11, 128, 136, 183). A probiotic *Lactobacillus* species, *L. helveticus,* has been shown to utilize constitutive surface layer proteins to inhibit *Escherichia coli* O157:H7 adherence to epithelial cells Johnson-Henry (63). Since we previously reported that *L. jensenii* inhibits gonococcal

adherence by a mechanism that does not involve the secretion of inhibitory substances (154), and we have shown that surface proteins are involved in the inhibition of gonococcal adherence, we next determined if surface components released from *L. jensenii* could inhibit gonococcal adherence to epithelial cells. Using a method published by Reid *et al.* (137) to isolate biosurfactants from lactobacilli, we isolated surface components released from lactobacilli and the biosurfactant activity of each preparation was tested by collapsed drop analysis. Each drop of RSC collapsed on the oily surface, demonstrating that RSC has biosurfactant activity, while the water controls remained beaded up on the oily surface.

The RSC was then tested for inhibitory activity against gonococcal adherence to epithelial cells by treating Hec-1-B cells for 3 h with varying concentrations of RSC prior to infection with gonococci in an adherence assay. The results of this experiment showed that gonococcal adherence was reduced by nearly 5-fold when the epithelial cells were treated with 1.7 mg/ml RSC (Figure 3.3). When the epithelial cells were treated with 0.42 mg/ml RSC, the gonococcal adherence frequency was inhibited less than 2-fold, and 0.17 mg/ml RSC had no effect on gonococcal adherence. The adherence of gonococci to the treated epithelial cells decreased as the amount of RSC increased, indicating that gonococcal adherence to epithelial cells is inhibited in a dose-dependent manner.

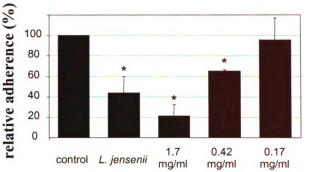

Figure 3.3. RSC inhibition of gonococcal adherence is dose dependent. Hec-1-B cells were incubated with either 1.7 mg/ml, 0.42 mg/ml, or 0.17 mg/ml RSC for 3 h before the addition of gonococci. Gonococcal adherence to epithelial cells was reduced to 43.9 ± 21.4% (*L. jensenii*, p= 0.01), 21.5 ± 14.4% (1.7 mg/ml RSC, p= 0.001), 65.1 ± 1.7% (0.42 mg/ml RSC, p= <0.001), and 95.6 ± 27.9% (0.17 mg/ml RSC, p= 0.81) of the untreated control. The data are averages of three independent experiments performed in triplicate. Error bars indicate standard error.

To rule out the possibility that the apparent reduction of gonococcal adherence to epithelial cells was caused by cell death or detachment caused by treatment with either RSC or bacteria, cell number and viability was assessed by trypan blue exclusion. There was no significant difference in the total number of nonviable cells (those that took up the trypan blue dye) after each treatment when compared to Hec-1-B cells with fresh medium (85.7 ± 22.2 blue cells per well), RSC (78.0 ± 11.3 blue cells per well, p = 0.704), lactobacilli (99.0 ± 16.0 blue cells per well, p = 0.552), gonococci (118.3 ± 35.1 blue cells per well, p = 0.368), lactobacilli + gonococci (73.7 ± 6.9 blue cells per well, p = 0.535), or RSC + gonococci (89.3 ± 12.2 blue cells per well, p = 0.857). Therefore, the effect on gonococcal adherence is not due cell death. When the number of live cells present in the cell monolayer (those that excluded the trypan blue dye) was compared after each treatment there was again no significant difference in cell counts when compared to Hec-1-B cells in fresh medium (7.83×10^4

$\pm$ 2.89 x 10^4 cells/well), *N. gonorrhoeae* (9.0 x 10^4 $\pm$ 4.0 x 10^4 cells/well, $p = 0.779$), *L. jensenii* (8.67 x 10^4 $\pm$ 1.89 x 10^4 cells/well, $p = 0.786$), RSC (9.67 x 10^4 $\pm$ 1.44 x 10^4 cells/well, $p = 0.533$), lactobacilli + gonococci (8.67 x 10^4 $\pm$ 1.89 x 10^4 cells/well, $p = 0.786$), or RSC + gonococci (9.33 x 10^4 $\pm$ 8.89 x 10^4 cells/well, $p = 0.589$). This suggests that any effects on gonococcal adherence frequency observed is due to an effect on gonococcal adherence to the epithelial cells, not an effect on the adherence of the epithelial cells to the cell culture plate.

Inhibition of gonococcal adherence to epithelial cells remains after removal of RSC. The RSC contains biosurfactant activity, which by definition are surface active molecules (139). Therefore, we hypothesized that a surface component of *L. jensenii* in the RSC inhibits gonococcal adherence by interacting with the surface of the epithelium. To test whether a component of the RSC interacts with the epithelial cell surface to inhibit gonococcal adherence, RSC, at 1.7 mg/ml, was incubated with Hec-1-B cells and then either washed away before infection with gonococci or left in the well during the infection. The relative adherence frequencies were then compared. There was no significant difference in the inhibition of gonococcal adherence when the RSC was removed (Figure 3.4, p =0.729). This suggests that the inhibitory component(s) of the RSC remain associated with the epithelial cells.

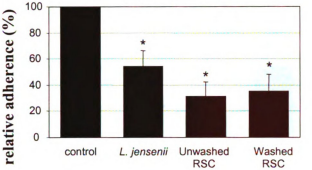

Figure 3.4. Washing of RSC-treated cells prior to inoculation with gonococci does not remove inhibitory activity. RSC was incubated with Hec-1-B cells for 3 h prior to infection with gonococci. Gonococcal adherence to epithelial cells was reduced to 53.3 ± 17.2% (*L. jensenii*, $p = 0.01$), 31.2 ± 15.0% (no wash, $p = 0.001$), 34.9 ± 18.1% (washed, $p = 0.003$). The data are averages of three independent experiments performed in triplicate. Error bars indicate standard error. Student's t-test p-value was 0.729 when washed RSC sample was compared to unwashed.

Characterization of the inhibitory components of the RSC. To determine if the inhibitory component(s) of the RSC was protein, an aliquot of RSC (1.7 mg/ml) was treated with ProK and incubated at 37°C for 2 h. A second aliquot was incubated at 37°C for 2 h as a negative control. Both aliquots were heat treated to inactivate the protease and centrifuged to remove any debris. These samples were then used to pre-treat Hec-1-B cells for 3 h before infection with gonococci (MOI 10). At 3 h post-gonococcal infection, the cells were lifted and plated to quantify adherence. Compared to the control, gonococcal adherence to RSC-treated cells was reduced 2-fold (Figure 3.5, $p = 0.004$). However, when the epithelial cells were pre-treated with ProK-RSC the gonococcal adherence frequency was similar to the adherence of the untreated control ($p = 0.755$). These results suggest that the inhibitory activity of the RSC is due to the presence of a protein(s).

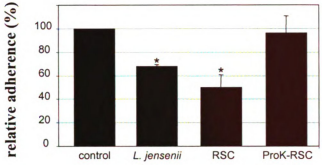

Figure 3.5. Proteinase K treatment of RSC abolishes inhibitory effect on *N. gonorrhoeae* adherence to Hec-1-B cells. RSC was incubated for 2 h at 37°C with proteinase K at 20 mg/ml. Gonococcal adherence to epithelial cells was reduced to 68.2 ± 2.0% (*L. jensenii*, $p<0.001$), 50.1± 14.6% (RSC, $p= 0.004$), 96.3 ± 18.9% (ProK treated RSC, $p= 0.755$). The data are averages of three independent experiments performed in triplicate. Error bars indicate standard error.

Purification of the RSC proteins. We next fractionated the inhibitory protein components of the RSC by anion exchange chromatography. The column was eluted with a gradient of 0-1 M NaCl and the proteins detected by A_{260}. A representative elution profile is shown in Figure 2.6A. Peak 1 (P1) eluted at 20 mM NaCl, and Peak 2 (P2) eluted at 1 M NaCl. SDS-PAGE analysis of samples from P1 and P2 showed there were several proteins in each pool (Figure 3.6B). The most abundant protein in P1 and the most abundant protein in P2, indicated in Fig. 3.6B, were excised and analyzed by tandem mass spectrometry. The protein from P1 had 2 peptides of observed mass over 1000 with 100% sequence identity to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Lactobacillus johnsonii* NCC 533. The protein from P2 had 8 peptides of observed mass over 1000 with 100% sequence identity to enolase from *Lactobacillus johnsonii* NCC 533 and *L. gasseri* 33323. P1 and P2 were dialyzed into PBS and then assayed for inhibitory activity. P1 (0.42 mg/ml) inhibited gonococcal adherence to epithelial cells by 2-fold. Similarly, P2 (0.42 mg/ml) also

inhibited

least tw

A

1

0.8

ABS260

0.6

0.4

0.2

0

6

B

C

inhibited gonococcal adherence by 2-fold (Figure 3.6C). This suggests there are at least two separable inhibitory components in the RSC.

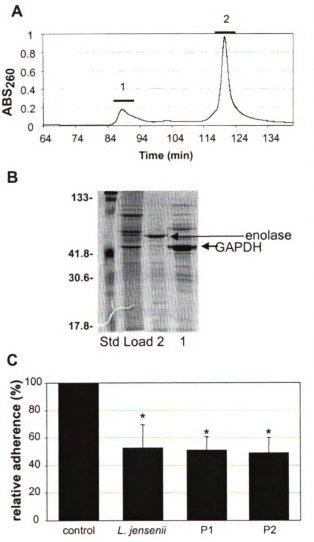

Figure 3.6. Fractionation of RSC by anion exchange chromatography. A. Elution profile of samples detected by UV absorbance (λ=260 nm) as the protein eluted from the column. Peaks 1 and 2 are marked P1 and P2. B. SDS PAGE analysis of P1 and P2. Long arrow points to the band with similarity to the enzyme enolase, and the short arrow points to the band with similarity to the enzyme GAPDH, as determined by MS/MS. C. Gonococcal adherence to epithelial cells pretreated with P1 and P2. Treatment of epithelial cells inhibited gonococcal adherence to 52.9 ± 23.9% (*L. jensenii*, p=0.027), 51.2 ± 14.0% (P1, p=0.004), and 48.8 ± 14.8% (P2, p=0.004). The data are the averages of three independent experiments performed in duplicate. Error bars indicate standard error.

To further separate the proteins in P2, the pool of protein was dialyzed against 50 mM Tris (pH 7.6), concentrated by ultrafiltration, and then separated by gel filtration size exclusion chromatography (Sephacryl S-200HR). Samples from fractions

containing protein were visualized by SDS-PAGE and assayed for biosurfactant activity. Fraction 49 was the only fraction that contained biosurfactant activity, and when visualized by SDS-PAGE, the sample contained a single band of protein. However, when assayed for inhibition of gonococcal adherence, this sample did not contain inhibitory activity. This suggests that the biosurfactant activity of the RSC is distinct and separable from the inhibitory activity.

Fraction 36, another fraction that contained a single protein band was tested for inhibitory activity. This fraction also did not inhibit gonococcal adherence to epithelial cells. Two other fractions assayed for inhibition of gonococcal adherence were fractions 24 and 29. These fractions were chosen because when visualized by SDS-PAGE, both fractions appeared to contain the putative enolase seen in Fig. 3.6B. Fraction 24 contained the putative enolase with an additional protein of 65.8 kdal, and reduced gonococcal adherence to epithelial cells to 26.5% of the no RSC control. Fraction 29 contained the putative enolase, the 65.8 kdal protein of fraction 24, and an additional protein of 30.6 kdal. This fraction reduced gonococcal adherence to 40.4% of the no RSC control. The level of inhibition appeared to correlate with the amount of the putative enolase in the fraction as seen by SDS-PAGE, with fraction 24 containing more of the putative enolase than fraction 29. The band correlating to the putative enolase was excised from an SDS-PAGE gel and verified by tandem mass spectrometry as having homology to enolase (7 peptides with m/z over 1000, with 100% sequence identity to enolase from *Lactobacillus gasseri* ATCC 33323).

Soluble fibronectin blocks inhibitory activity of the RSC. Gonococci are known to bind to fibronectin (Fn) during the invasion of epithelial cells (174). Since enolase of

other *Lactobacillus* species has been shown to bind Fn (7, 26, 70) and a putative enolase is the main component of one of the inhibitory fractions of the RSC, we hypothesized that the RSC might reduce gonococcal adherence to epithelial cells by blocking gonococcal binding to extracellular matrix components such as fibronectin. To test this, RSC was treated with increasing amounts of soluble Fn prior to addition to the epithelial cells. After 3 h, the RSC-Fn solution was removed and the cells were infected with *N. gonorrhoeae*. When no Fn was added, 0.42 mg/ml RSC reduced gonococcal adherence to 40.4 ± 17.0%. When the RSC was treated with either 2.5, 5, or 7.5 μM Fn, gonococcal adherence was reduced, but as the amount of Fn increased, the inhibitory activity of the RSC was reduced, to 54.9 ± 8.0%, 70.1 ± 8.7%, and 74.6± 9.9%, respectively (Figure 3.7). When the RSC was treated with 10 μM soluble Fn, the gonococcal adherence frequency was 95.3 ± 7.5% relative to the control, essentially no different from gonococcal adherence in the absence of RSC. This is consistent with the hypothesis that the mechanism of RSC inhibition of gonococcal adherence is due to an inhibition of gonococcal interactions with Fn on the cells.

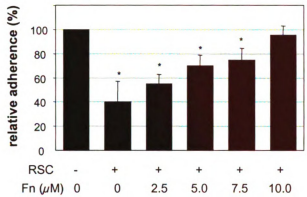

Figure 3.7. Gonococcal adherence to cells treated with RSC that had been pre-treated with increasing amounts of soluble fibronectin. Gonococcal adherence frequencies are reported as relative to a no RSC-no Fn control and Student's t-tests were used to compare each Fn treatment with no Fn treatment. Adherence to epithelial cells was reduced to 40.4 ± 17.0% (0.42 RSC), 54.9 ± 8.0% (RSC-2.5 μM Fn, p= 0.374), 70.1 ± 8.7% (RSC-5.0 μM Fn, p= 0.121), 74.6 ± 9.9% (RSC-7.5 μM Fn, p= 0.091) and 95.3 ± 7.5% (RSC-10 μM Fn, p= 0.019). The data are averages of three independent experiments performed in duplicate. Error bars indicate standard error.

Effect of His_6-Eno on gonococcal adherence to Hec-1-B cells. One of the two proteins of the RSC that co-purified with the inhibitory activity had significant similarity to an enolase from *Lactobacillus gasseri.* To determine if this protein is the inhibitory component, the enolase gene from *L. jensenii* ATCC 25258 was recombinantly expressed as a His_6-tagged protein in *E. coli* and purified by chromatography (Figure 3.8A). The purified protein was then used at various concentrations to treat epithelial cells prior to gonococcal infection. His_6-Eno inhibited gonococcal adherence to epithelial cells at all concentrations tested by 50-80% (Figure 3.8B). As the concentration of His_6-Eno increased, the adherence of gonococci to epithelial cells decreased, again indicating a dose-response.

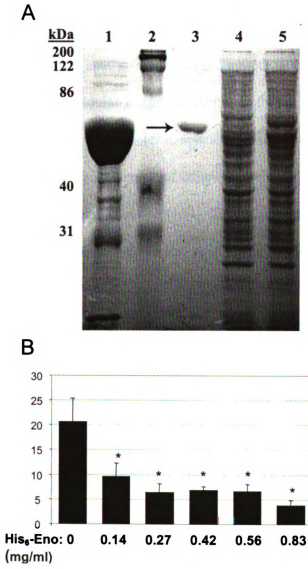

Figure 3.8. Purification of His_6-Eno and its effect on gonococcal adherence to Hec-1-B cells. A. SDS-PAGE gel analysis of His_6-Eno. 18 μg purified His_6-Eno (lane 1), MW standards (lane 2), 0.72 μg purified His_6-Eno (lane 3), uninduced *E. coli* BL21λDE3 pET24a-eno (lane 4), induced *E. coli* BL21λDE3 pET24a-eno (lane 5). Arrow indicates His_6-Eno. B. Gonococcal adherence to cells pre-treated with His_6-Eno. Gonococcal adherence in the absence of His_6-Eno: 20.1 ± 4.0%; in the presence of 0.14 mg/ml His_6-Eno: 10.4 ± 2.8%, p= 0.028; 0.27 mg/ml His_6-Eno: 6.5 ± 1.8; p= 0.013; 0.42 mg/ml His_6-Eno: 6.9 ± 0.7%, p= 0.004; 0.56 mg/ml His_6-Eno: 6.7 ± 1.4%, p= 0.013; 0.83 mg/ml His_6-Eno: 3.8± 1.0%, p= 0.006. The data are averages of three independent experiments performed in duplicate. Error bars indicate standard error.

DISCUSSION

Adherence is a critical first step in the infection of a new host for many pathogens, including *N. gonorrhoeae.* As an incoming pathogen, gonococci have to overcome the host's defenses and compete with the indigenous microbiota to effectively colonize the endocervical epithelia. *Lactobacillus jensenii*, one of the most commonly isolated bacterial species from the healthy human vaginal tract, has been shown to inhibit gonococcal adherence to and invasion of epithelial cells in a co-culture model of infection (154). This inhibition of gonococcal adherence was not caused by any secreted factor, such as hydrogen peroxide, due to co-aggregation between the gonococci and the lactobacilli, or competition for receptors. This ability to inhibit gonococcal adherence, without direct killing of the pathogen, might be specific to *Lactobacillus* species, as we see no inhibition of adherence when cells are pre-treated with another Gram positive bacterium, *Bacillus subtilis*. This finding correlates with research which showed vaginal *Lactobacillus* isolates inhibit gonococcal adherence to the cell line ME180 better than intestinal isolates (179), suggesting that this inhibitory mechanism may have developed due to competition in the vaginal ecosystem. In this work, we have examined the components of lactobacilli, gonococci, and the epithelial cells involved in an effort to elucidate the mechanism(s) of this inhibition.

In our *in vitro* model of gonococcal infection, pre-colonization of glutaraldehyde-fixed epithelial cells with *L. jensenii* reduced gonococcal adherence by 2-fold (Figure 3.1B), suggesting that *L. jensenii* inhibits gonococcal adherence by a mechanism that does not require the epithelial cells to be metabolically active. Since

lactobacilli inhibit gonococcal adherence to epithelial cells by a mechanism that does not require a response from the epithelial cells, this suggests that a component of *L. jensenii* is important for the interaction. Our results show that methanol-fixed *L. jensenii* inhibited gonococcal adherence to epithelial cells to a similar extent as live lactobacilli (Figure 3.2). Therefore, it is likely that the components of lactobacilli necessary to inhibit gonococcal adherence to epithelial cells are surface associated. Treatment of methanol-fixed *L. jensenii* with proteinase K abolished the inhibition, indicating that the inhibitory factor is a protein or contains a protein component.

N. gonorrhoeae utilizes a type IV pilus for adherence to epithelial cells in culture (104, 160) and this adhesin has been shown to be essential for gonococci to colonize human volunteers (74, 161). Therefore, pilus-mediated adherence would be a likely target for the observed *Lactobacillus* inhibition. However, when the effect of *L. jensenii* pre-colonization of epithelial cells on the adherence of piliated gonococci and non-piliated gonococci was examined, both piliated and non-piliated gonococci were inhibited to a similar extent suggesting that the mechanism of inhibition is not specific to the main gonococcal adhesin, type IV pili, but is a more nonspecific mechanism.

One mechanism that could explain this global inhibition of gonococcal adherence would be the production of a biosurfactant by the lactobacilli. Biosurfactants are amphipathic molecules produced by microorganisms that serve a variety of purposes, including adsorption to surfaces (139). It is possible that *L. jensenii* produces a biosurfactant that adheres to the epithelial cell surface, which is left behind when the bacterium desorbs. The molecule left on the epithelial cell surface could change the nature of the surface such that it is less able to support gonococcal adherence. Over 15

species of lactobacilli have been shown to produce biosurfactants (137). One of the most well characterized *Lactobacillus*-produced biosurfactants is produced by *L. reuteri* RC-14, and was found to be effective in preventing the adherence of *Enterococcus faecalis* to hydrophilic glass and silicone rubber surfaces (55, 175, 177). The biosurfactant from *L. reuteri* RC-14 was also found to inhibit abscess formation by *Staphylococcus aureus* in a surgical implant model in rats, demonstrating that biosurfactants can inhibit pathogen adherence to biotic surfaces as well as abiotic surfaces (51). Furthermore, a surface-associated protein was purified and identified as the inhibitory component from this biosurfactant (55). This protein was identified as a collagen-binding protein, however, it was not demonstrated if this protein alone had biosurfactant activity.

Using a method to isolate biosurfactants, *L. jensenii* was caused to release surface proteins. The released surface component mixture (RSC) has surfactant activity as demonstrated by collapsed drop analysis and also inhibited gonococcal adherence in a dose-dependent manner (Figure 3.3). However, when the RSC was fractionated by column chromatography, the inhibitory activity was separated from the biosurfactant activity. A cell-free protein extract from *L. helveticus*, without biosurfactant activity, has also been found to inhibit pathogen adherence to epithelial cells (63). This preparation was an extract of surface layer proteins. Surface layer proteins are a paracrystalline layer associated with the outer surface of some bacteria, and play a role in adhesion to surfaces (63). However, we do not consider surface layer proteins as a potential inhibitory factor, since *L. jensenii* does not produce surface layer proteins (129).

In the RSC there are at least two inhibitory components, since two distinct inhibitory fractions were isolated from the RSC following anion exchange chromatography. The first fraction contains several proteins shown by SDS-PAGE (Figure 3.6B), of which the major band has been identified by MS/MS as a putative glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The second fraction was analyzed further and an inhibitory protein was identified as a putative enolase by MS/MS. We subsequently cloned the enolase gene from *L. jensenii* and purified the recombinant His_6-tagged protein tested it for ability to inhibit gonococcal adherence to epithelial cells. Since His_6-Eno was able to inhibit the adherence of *N. gonorrhoeae* (Figure 3.8B), we can conclude that the putative enolase was one of the inhibitory factors present in the RSC.

While both GAPDH and enolase are normally thought of as cytosolic enzymes involved in glycolysis, the enzymes have both been found surface associated in several *Lactobacillus species* (8, 26, 78). Furthermore, lactobacillar enolase has been shown to bind to extracellular matrix components such as fibronectin, collagen, and laminin (7, 70, 144). Our data shows that pre-incubation of the RSC with soluble fibronectin before use in gonococcal adherence assays abolished the inhibitory effect of the RSC in a dose dependent manner (Figure 3.7), consistent with the hypothesis that *L. jensenii* inhibits gonococcal interactions with epithelial cells by occluding fibronectin binding sites with its enolase. In the vaginal tract, fibronectin is secreted and coats the mucosal surface (32), indicating the hypothesis that a surface component of *L. jensenii* binds to fibronectin to prevent gonococcal adherence is biologically relevant. Future experiments will be necessary to determine if the putative enolase is in fact the

fibronectin binding protein and whether the enzymatic activity is necessary for the inhibition.

While a putative GAPDH was identified as the most abundant protein in P1 from the RSC (Figure 3.6C), it still has not been identified as the inhibitory component of that fraction. Since GAPDH from other *Lactobacillus* species is known to bind colonic mucin and fibronectin (144), this protein is a candidate for an inhibitor of gonococcal adherence to epithelial cells, however, this hypothesis remains to be tested.

In summary, we have shown that *Lactobacillus jensenii* inhibits gonococcal adherence to epithelial cells independently of an epithelial cell response, utilizing at least one surface associated protein. When removed from the surface of lactobacilli, these proteins can still inhibit gonococcal adherence to epithelial cells, however inhibition is abolished by treatment with fibronectin, suggesting the inhibitory protein blocks gonococcal binding to this extracellular matrix component. Now, with gonococci having achieved "superbug" status it is imperative that new methods of treatment and prevention be discovered that do not rely solely on antibiotics. The potential use of *Lactobacillus* products for prevention of gonorrhea is an exciting possibility that needs to be further explored.

CHAPTER FOUR

Spurbeck R.R. and C.G. Arvidson. (2010). *Lactobacillus jensenii* utilizes an enolase to inhibit *Neisseria gonorrhoeae* Adherence to Epithelial Cells.

Abstract

Recently a surface-associated protein produced by *Lactobacillus jensenii* ATCC 25258 with homology to enolase was found to inhibit the adherence of the sexually transmitted pathogen, *Neisseria gonorrhoeae*, to epithelial cells in culture. Here, we show that protein, now called gonococcal anti-adherence protein 1 or GAP1, is an active enolase *in vitro*. A recombinantly expressed, epitope-tagged version of the protein, His_6-GAP1, inhibited gonococcal adherence utilizing the substrate-binding site, but does not require the activity for inhibition. Furthermore, an *L. jensenii gap1*$^-$ strain carrying an insertional mutation in the *L. jensenii* GAP1 gene, exhibited reduced inhibition of gonococcal adherence consistent with this being a multifactorial process.

Introduction

Pathogenic and commensal bacteria are known to produce "anchorless" proteins that are associated with the cell surface, but have no known mechanism for export or attachment (7). One such protein is the enzyme enolase. Usually considered to be a cytosolic enzyme, enolase catalyzes the conversion of 2-phosphoglycerate (2PGA) into phosphoenolpyruvate (PEP) through the removal of H_2O, a key step in glycolysis. Enolase can also be thought of as a "moonlighting enzyme," or a protein that performs more than one function for the bacterium (57). Several pathogens including *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* (7), as well as the fungal pathogen *Paracoccidiodes brasiliensis*, the protist *Trichomonas vaginalis,* and the filiarial parasite *Onchocerca volvulus*, utilize this enzyme to interact with the host (37, 66, 112). Specifically, these pathogens use their surface-associated enolases to bind plasminogen, which allows for efficient cleavage by plasminogen activators to the proteolytic form, plasmin (7, 37, 66, 112). Once active, plasmin degrades extracellular matrix proteins, allowing the pathogens to invade deeper into the host tissues (7, 37, 66, 112).

Surface-associated enolases have also been found in several species of *Lactobacillus*, usually along with cytoplasmic counterparts. For example, *L. johnsonii* was shown to express three distinct enolase proteins (7). The enolases from both *L. johnsonii* and *L. crispatus* bound plasminogen thereby enhancing the activation to plasmin *in vitro* (7). However, these organisms are rarely associated with disease, and are present in the human gastrointestinal and vaginal tracts of healthy individuals. The reason these organisms have surface-associated enolase able to bind plasminogen has

not been elucidated. However, lactobacillar enolases could allow the bacteria to colonize the mucosal epithelium, since they are also known to bind to extracellular matrix proteins, such as laminin, collagen, and fibronectin (7, 26).

We have recently shown that *Lactobacillus jensenii* can inhibit the adherence of *Neisseria gonorrhoeae* to epithelial cells by surface associated proteins (154-155). A preparation of released surface components (RSC) was able to inhibit gonococcal adherence to epithelial cells, and a protein with homology to enolase 3 (Eno3) of *L. johnsonii* correlated to the inhibitory activity. Here, we examine the role of this protein, GAP1, as an inhibitor of gonococcal adherence using insertional mutagenesis in conjunction with biochemical characterization of a recombinantly expressed, epitope-tagged enolase protein.

Materials and Methods

Bacterial strains and culture conditions. *N. gonorrhoeae* MS11 [P^+, Tr] (148) was grown at 37°C in a humidified 5% CO_2 environment (+ CO_2) on GC agar (Acumedia, Lansing, MI) with supplements (75) and VCNT inhibitor (GCV, Becton, Dickinson, and Company, Sparks, MD). *N. gonorrhoeae* MS11-911 ['*lacZ*-Em^R] was grown at 37°C + CO_2 on GC agar with supplements, VCNT inhibitor, and erythromycin at 3 mg/L (Erm^3). *L. jensenii* WT (ATCC 25258, wild type) was grown at 37°C + CO_2 on MRS agar (Becton, Dickinson, and Co). *L. jensenii-gapl*$^-$ (ATCC 25258, *gapl*$^-$) was grown at 37°C + CO_2 on MRS agar with erythromycin at 5 mg/L (Erm^5). Both *Escherichia coli* strain BL21λDE3 containing pET24a-eno and *E. coli* EC101 were maintained on LB agar with kanamycin at 100 mg/L, (Kan^{100}) at 37°C + CO_2. *E. coli* EC101+ pORI19 was maintained on LB agar with erythromycin at 300 mg/L (Erm^{300}) at 37°C + CO_2. *L. jensenii*+ pVE6007 was maintained on MRS agar with chloramphenicol at 5 mg/L (Cm^5) at 35°C + CO_2.

Cell culture. Hec-1-B cells (ATCC HTB-113), were cultured in Dulbecco's Modified Eagle Medium (DMEM with high glucose and L-glutamate, Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS, Invitrogen) at 37°C + CO_2. Adherence assays were carried out as described previously in 24 well cell culture plates in which the Hec-1-B cells were grown to 50-90% confluency (Spurbeck, 2010).

Protein expression and purification. His_6-GAP1 was expressed in *E. coli* BL21λDE3 and purified as previously described (155). Purified protein was dialyzed against 1x phosphate buffered saline (PBS, pH 7.0) before use in cell culture and enzyme assays.

Insertional mutagenesis. A mutant was constructed in *L. jensenii* 25258 using the method described by Whitehead et al (185) that has been used to generate insertional mutations of *Lactobacillus reuteri,* with some modifications. The PCR product from the GAP1 gene was digested with *Eco*RI and a 400 bp fragment was ligated into pORI19 (Em^R), which replicates in *E. coli* and *Lactobacillus* only if the strain contains RepA, and transformed into *E. coli* EC101 (*rep*A^+). Transformants were screened on plates containing X-Gal (40mg/l) for insertion within the *lacZ* gene of pORI19, and confirmed by PCR. The resulting plasmid, pRRS2-37, was then electroporated (2500 mV, 25 μF, 400 Ω) into *L. jensenii* 25258 containing pVE6007 (Cm^R, *repA*$^+$), which is temperature-sensitive for replication. Electroporation and preparation of electrocompetent *L. jensenii* was carried out as described in Papagianni et al. (125) with the following modifications. The lactobacilli were grown overnight in 4 x 8 ml/tube of MRS broth at 37°C + CO_2. A 1:5 dilution was made into pre-warmed MRS without mixing. The lactobacilli were incubated at 30°C + CO_2 for 2 h until the O.D. reached 0.5. 200 μl penicillin G (10 mg/ml) was added and the culture was incubated an additional h at 30°C + CO_2. The cells were then harvested by centrifugation and the supernatant was removed. The cells were resuspended in 1 ml

LiAc solution (100 mM LiAc; 10 mM DTT; 0.6 M sucrose; 10 mM Tris-HCl, pH 7.5; filter-sterilized) and incubated 1 h at room temperature (RT). The cells were washed twice with deionized H_2O, then with 50 mM EDTA, deionized H_2O, and 0.3 M sucrose. The cells were then resuspended in 0.3 M sucrose and placed on ice for at least 30 min before electroporation (protocol provided by Dr. Lin Tao, University of Chicago, personal communication). Transformants were selected on MRS agar (Cm^5Erm^5) at 35°C + CO_2. Two transformants were grown in MRS broth (Cm^5Erm^5) for 18 h without shaking at 35°C + CO_2. To select for integration of pRRS2-37 into the *Lactobacillus* chromosome, the culture was diluted 1:200 and grown in MRS Erm^5 broth for 8 h at 42°C under anaerobic conditions. Serial dilutions were then plated on MRS Erm^5 agar and incubated at 42°C + CO_2 for 24-48 h to select for a single cross-over event with pRRS2-37 inserted into the chromosome at the region of sequence similarity to the gene fragment on the plasmid. Erm^R isolates were screened for Cm^R at 35°C + CO_2 to ensure loss of pVE6007. To confirm the placement of pRRS2-37 on the *L. jensenii* chromosome and to show that the gene encoding GAP1 was no longer intact, genomic DNA from the isolates was subjected to PCR using a primer specific for the gene (EnoFwd) and another specific to pORI19 (M13R, Figure 4.1).

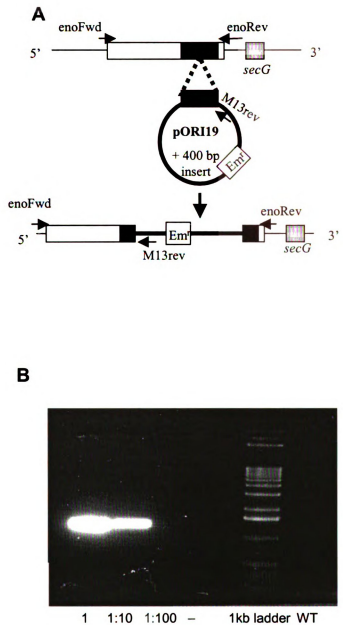

Figure 4.1. Insertional mutagenesis of *L. jensenii* 25258 *eno*. A. Schematic diagram of plasmid integration into chromosomal locus to disrupt *eno*. Primers used to amplifiy regions to confirm construct are indicated. B. PCR amplification to verify insertion. Genomic DNA from $Erm^{R}Cm^{S}$ transformants was used as template for PCR using primers enoFwd and M13rev. Templates: 1 = undiluted mutant genomic DNA, 1:10 = mutant genomic DNA, 1:100 mutant genomic DNA, - = no template control, and WT = *L. jensenii* WT genomic DNA.

Production of released surface components (RSC). RSC was produced from 1 L overnight cultures of *L. jensenii* or *L. jensenii-gap1*$^-$ as described previously (155). The Bradford method was used to determine the protein concentration for each RSC preparation (Bio-Rad Laboratories) and the RSC was tested for inhibitory activity against gonococcal adherence to Hec-1-B cells as described below.

Whole cell enolase activity assays. Whole cell enolase activity assays were carried out as described by Pancholi and Fischetti (124), with some modifications. Briefly, *L. jensenii* WT and *L. jensenii gap1*$^-$ were grown overnight at 37°C. Various amounts of bacteria were harvested by centrifugation, washed with 100 mM HEPES buffer, pH 7, and then resuspended in 100 mM HEPES, pH 7, with 10 mM $MgCl_2$ and 7.7 mM KCl with or without substrate (3 mM 2PGA). The bacterial suspensions were incubated 5 min at 37°C and then the bacteria were removed by centrifugation (6000 x g for 5 min). PEP production was determined spectrophotometrically ($\lambda = 240$ nm) using the extinction coefficient of PEP of 2.6×10^3 M.

His_6-GAP1 activity assays. Enolase activity of purified His_6-GAP1 was assayed as described (124) with some modifications. 5 μg of protein was incubated with various concentrations of 2PGA (0.5-3mM) in the reaction buffer described above (100 mM HEPES buffer, pH 7, 10 mM $MgCl_2$, 7.7 mM KCl). All reactions were carried out at RT and A_{240} was measured every 5-10 s to determine enzyme kinetics. Values were corrected by subtracting out the absorbance recorded for reactions without substrate. The effects of the known enolase inhibitors, $CaCl_2$ (Ca^{2+}) and an equimolar solution

of NaF and NaH_2PO_4, pH7.0 (Fl^-+P_i), on the activity of His_6-GAP1 was also tested at various concentrations as described in the text.

PCR amplification of cytosolic enolase. PCR amplification of *gap1* was conducted using primers enoFwd and enoRev, while amplification of *eno2* used 0838Fwd and 0838Rev. Genomic DNA was isolated from *L. gasseri* 33323 and served as a positive control for the presence of both genes. Genomic DNA from both *L. jensenii* WT and *L. jensenii gap1*$^-$ was isolated and amplified to determine if both genes were present. pET24a-eno served as a negative control for the amplification of *eno2*.

Statistical analysis. All data were analyzed by using an unpaired Student's t-test. A *p*-value below 0.05 was considered statistically significant and is indicated by an asterisk (*) in the graphs.

Results

DNA sequence analysis of *L. jensenii* ATCC 25258 *gap1*. We previously cloned the *gap1* gene from *L. jensenii* 25258 by amplification of the ORF and the intergenic 3' sequence to the neighboring gene, *secG* (155). This sequence is 99% identical to the gene number 1305 of *L. gasseri* 33323. This species of lactobacilli also inhibits gonococcal adherence to epithelial cells (154). *L. jensenii* 25258 *gap1* is also 92% identical to that of *L. johnsonii* NCC533 *eno3*, which has been well characterized (7). However, it is only 83% identical to the putative enolase gene sequences from either *L. jensenii* 1153 or JV-16.

At the amino acid level, *L. jensenii* 25258 GAP1 contains all of the conserved amino acids necessary for metal binding (4/4), substrate binding (7/7), and the dimer interface present in all characterized enolases (37/37) (Figure 4.2A). The protein is also predicted to contain the characteristic TIM β-barrel domain which is found in all of the enzymes in the enolase superfamily (shaded in gray in Figure 4.2A). A phylogenetic tree depicting the homology to known enolase protein sequences in other lactobacilli and Gram positive pathogens is shown in Figure 4.2B. *L. jensenii* 25258 GAP1, *L. gasseri* 33323 gene 1305 product, and Eno3 from *L. johnsonii* form a divergent group from other lactobacillar enolases, and tend to be more similar to the enolases known to be surface-associated in *L. plantarum* and in Gram-positive pathogens. Within the TIM β-barrel domain, there are 19 residues found only in the group of GAP1-like sequences (highlighted in bold, Figure 4.2A). These amino acid changes could be significant in substrate binding or the conformation of the active site; however, this has yet to be addressed experimentally.

A

```
1   MSVITDIHAR EVLDSRGNPT AEAEVYTELG GFGRAIVPSG ASTGEHEAVE LRDGDKSRFG
          ^ ^ ^^^^^^^^    ^          ^         #
61  GQGVLTAVEN VNGEIAKAVI GLDVTDQRLI DQTMIDLDGT PNKGRLGANA ILSVSLASAR

121 AAADELGLPL YEYLGGPNAH VLPTPMMNVI NGGKHADNNV DIQEFMIMPV GAKSLHEAVR
                                    **^^^                    ^^
181 MGAETFHTLK GLLQERGEST AVGDEGGFAP NLKNNEEPFE ILVEAIQRAG YKPGQDIAIA
     ^^  ^^              ^^  *   ^  ^
241 FDCAASEFYN KDTKKYVTVA DGREYTAEEW TSLIEDLVDK YPVISVEDPL DENDWEGWKT
     #                                              #
301 FTERLGDKVQ IVGDDLFVTN TSYLEKGIKM GVANSILIKL NQIGTLTETF EAIEMAKEAG
               #                          *
361 YTAVVSHRSG ETEDTTIADL VVATNAGQIK TGSMSRTDRI AKYNQLMRIE EALGSTAQYK
          ***  ^^^                   *       ^^^ ^ ^  ^  ^^  ^
421 GIHSFYNLHK QF 432
```

B

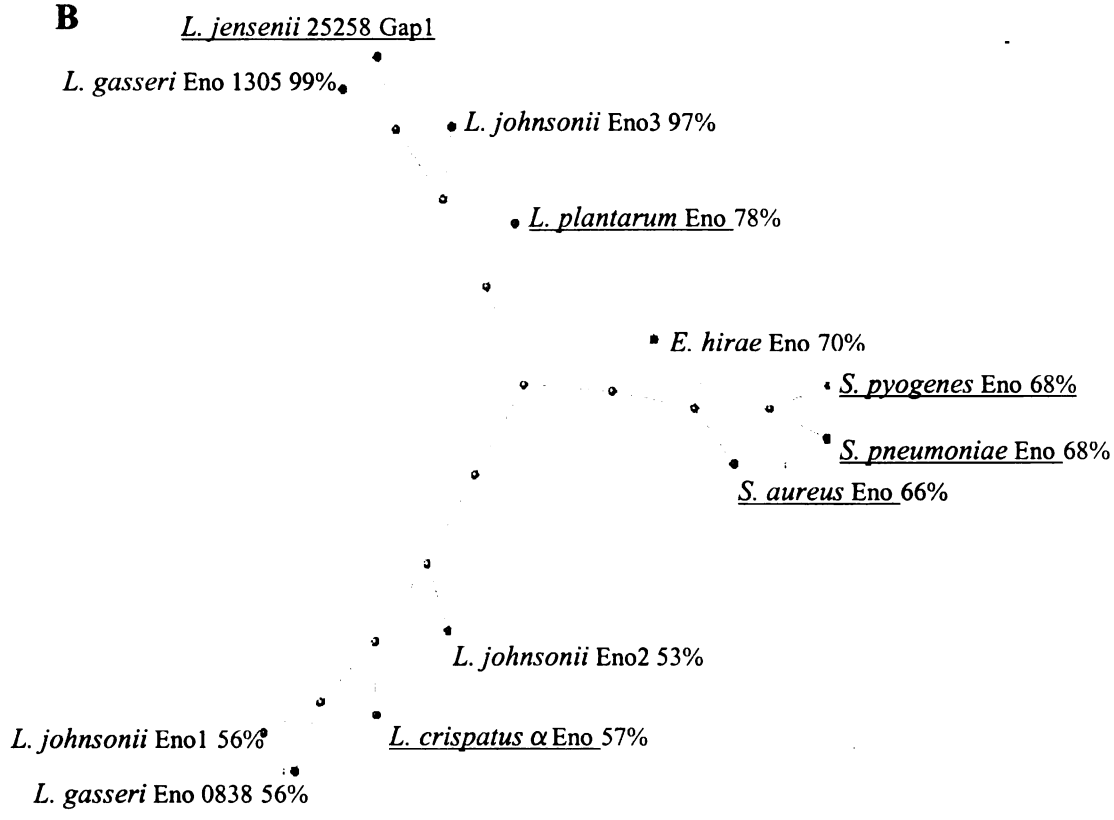

Figure 4.2. GAP1 protein characteristics. A. The primary amino acid sequence of *L. jensenii* 25258 GAP1. The region shaded in gray corresponds to the TIM β-barrel domain common to enolases (16). Amino acids of interest that are specific to GAP1 are highlighted in bold font. The conserved amino acids are marked below the residue with # for metal binding, * for substrate binding and ^ for dimer interface. B. A phylogenetic tree of enolase protein sequences. % = identity to GAP1. Distances were calculated using the Grishin model with maximum sequence difference of 0.85 (142).

Another region of interest is the C-terminus of the protein, where 7 of the last 14 amino acids are found only in the enolases in the GAP1-like group and the pathogen group and 4 of the 14 are found only in the GAP1-like group. Recently, two C-terminal lysine residues of streptococcal surface enolase were shown to have a role in the binding of plasminogen, suggesting that this region might be important for binding of alternative substrates (35).

Enolase activity of His_6–GAP1 in the presence and absence of inhibitors. Enolase activity assays were carried out using the purified recombinant protein, His_6–GAP1, to determine if the tagged protein was enzymatically active. His_6–GAP1 converted 2PGA to PEP, and the activity correlated with the amount of enzyme, suggesting that the reaction is a first order reaction. Furthermore, when the substrate concentration was varied, the reaction followed Michaelis-Menten kinetics. K_m for 2PGA was 92.9 ± 0.82 μM and the maximum velocity was determined to be 791.6 ± 297.5 μM min^{-1} $(mg\ protein)^{-1}$. $Fl^- + P_i$ is known to complex with the Mg^{2+} ions present in the active site of the enolase, causing a conformational change that closes the active site (87). When added to the reaction at 5 mM, $Fl^- + P_i$ reduced the enolase activity of His_6–GAP1 by 87.2%. Ca^{2+}, another inhibitor of enolase activity, binds with higher affinity than Mg^{2+} to the metal binding sites in the active site, leaving the active site accessible to substrate, but blocking transformation of the substrate (86). When added to the reaction in increasing amounts, Ca^{2+} reduced 2PGA transformation. At 2.26 fold more

Ca^{2+} than Mg^{2+} in the reaction, the enolase activity of His_6–GAP1 was reduced by 87.8%.

Effect of enolase inhibitors on His_6–GAP1 reduction of gonococcal adherence.

We previously showed that His_6–GAP1 concentrations as low as 0.14 mg/ml inhibited gonococcal adherence to epithelial cells by ~50% (155). This inhibition is specific to His_6–GAP1, since incubation of the epithelial cells with BSA (1.76 mg/ml) prior to gonococcal inoculation did not affect the adherence of gonococci (BSA: 19.3 ± 6.7%, control: 14.7 ± 8.5%, $p = 0.607$). However, it was not clear whether enzymatic activity of the enolase was required. The enolase inhibitors Fl^-+P_i and Ca^{2+} were examined for their effect on His_6–GAP1 inhibition of gonococcal adherence to epithelial cells. Glutaraldehyde fixation was done to ensure that the results observed were due solely to effects of the inhibitors on the enzyme, and not due to any change in the epithelial cells. To make certain that there were no direct effects of the inhibitors or enzyme on *N. gonorrhoeae*, His_6–GAP1 and inhibitors were removed and the fixed cells were washed with PBS prior to introduction of gonococci. Surprisingly, Fl^-+P_i abolished the inhibition of gonococcal adherence to epithelial cells, such that gonococci adhered essentially the same as in the absence of His_6–GAP1. However, addition of Ca^{2+} did not affect the reduction of gonococcal adherence by His_6–GAP1 (Figure 4.3). Incubation of the enzyme with additional Mg^{2+}, the metal cofactor necessary for enolase enzymatic activity, also had no effect on the inhibition of gonococcal

adherence (data not shown). Since Ca^{2+} blocks enolase activity by replacing the catalytic Mg^{2+} ions without occluding the substrate binding site, and $Fl^{-}+P_i$ complexes with the structural Mg^{2+} ions closing the substrate binding site, these data suggest that the enolase activity is not necessary but the substrate binding site is essential for inhibition of gonococcal adherence to epithelial cells to occur.

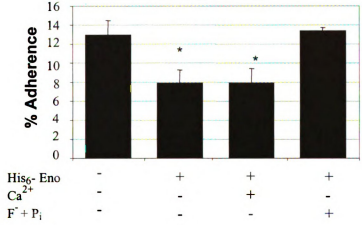

Figure 4.3 His_6-Eno inhibition of gonococcal adherence to glutaraldehyde fixed Hec-1-B cells. *N. gonorrhoeae* alone adhered to the fixed cells with a frequency of 12.9 ± 1.6%. When treated with 0.14 mg/ml His_6-Eno, gonococcal adherence was reduced to 7.9 ± 1.3% (no inhibitors, $p = 0.036$), 7.9 ± 1.5% (Ca^{2+}, $p = 0.043$), or 13.4 ± 0.4% (F^{-} + P_i, $p = 0.746$). The data are the averages of 3 independent experiments performed in duplicate. Error bars depict standard error.

Construction of an enolase mutant. To determine if GAP1 is one of the inhibitory components involved in the inhibition of gonococcal adherence to epithelial cells by live *L. jensenii*, the encoding gene was insertionally inactivated on the chromosome of *L. jensenii* 25258. Growth of *L. jensenii gap1*$^{-}$ was not significantly different than that of *L. jensenii* WT (data not shown). By sequencing pRRS2-37, it was determined that the plasmid inserted at bp 1252, disrupting the last 42 bases in the *gap1* gene sequence

(Figure 4.1A). The insertion occurred between amino acid 417A and 418Q in the protein sequence, 15 residues from the C-terminus.

Enolase activity of intact *L. jensenii* and cytoplasmic fractions. To determine the effect of the *gap1*⁻ mutation on cellular enolase activity, we used an activity assay (124) using whole, intact lactobacilli to catalyze the reaction. As seen in Figure 4.4A, intact *L. jensenii* 25258 have enolase activity that increases as the cell concentration increases. However, when *gap1* has been inactivated, as in *L. jensenii gap1*⁻, enolase activity is undetectable. From this result, we conclude that the gene that was inactivated in *L. jensenii gap1*⁻ encodes an enolase present on the surface of the lactobacilli. Since bacteria typically have cytoplasmic enolases, we also assayed cytoplasmic extracts of both *L. jensenii* WT and *L. jensenii gap1*⁻. Accordingly, lactobacilli were sonicated to disrupt the cells, and then centrifuged to remove the insoluble fraction and any unbroken cells (including the surface-associated GAP1). The protein content of the soluble fraction was determined and then assayed for enolase activity at various protein concentrations. The results of these experiments showed that cytoplasmic extracts of both *L. jensenii* WT and *L. jensenii gap1*⁻ have enolase activity that increases with protein concentration, suggesting that there is another enolase that is strictly intracellular (Figure 4.4B). However, the enolase activity in the cytoplasm of *L. jensenii gap1*⁻ is significantly lower than that of *L. jensenii* WT (50 μg: $p = 0.013$, 75 μg: $p = 0.015$, and 100 μg: $p = 0.002$) which suggests that surface-associated enolase is either active intracellularly or some of the GAP1 on the surface of *L. jensenii* WT is still present in the wildtype samples.

Due to the similarity between the *L. jensenii gap1* and *L. gasseri* 33323 gene 1305, we hypothesized that if a second enolase gene was present in *L. jensenii*, that it would be similar in sequence to that of *L. gasseri* 33323 gene 0838. Therefore, we constructed primers specific to *L. gasseri* 33323 gene 0838 that do not cross react with *gap1*. By PCR, we confirmed the presence of a second enolase, and that the primers do not amplify GAP1 (data not shown). This result suggests that *L. jensenii* has evolved to have two enolases, one that works intracellularly, and a second that has been adapted to serve an alternate function on the bacterial cell surface.

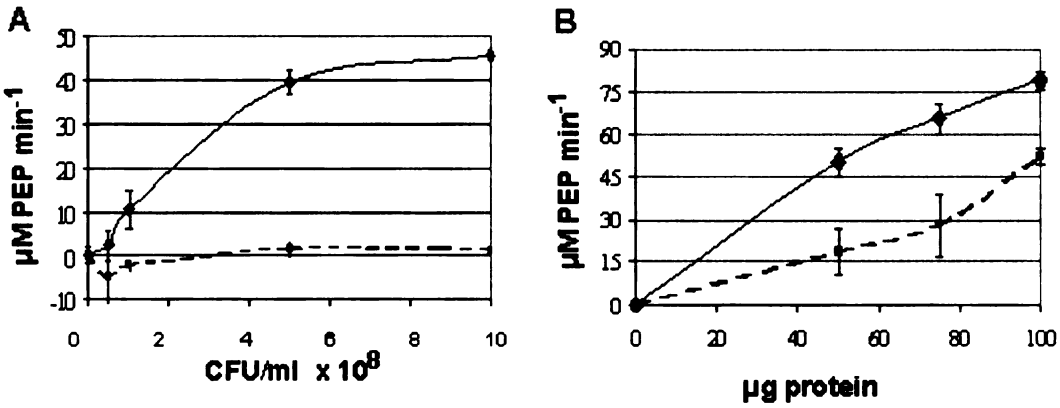

Figure 4.4. Enolase activity of *L. jensenii* WT and *L. jensenii gap1*⁻. A. Enolase activity of increasing concentrations of intact lactobacilli plotted as a function of PEP produced. B. Enolase activity of increasing amounts of cytoplasmic extracts from *L. jensenii* WT and *L. jensenii gap1*⁻. Solid line = *L. jensenii* WT and the dotted line = *L. jensenii gap1*⁻. The data are the averages of 3 independent experiments performed in triplicate. Error bars depict standard error.

Effect of *gap1*⁻ on gonococcal adherence to epithelial cells. To examine the effect of *L. jensenii gap1*⁻ on gonococcal adherence, Hec-1-B cells were colonized with either *L. jensenii* WT, or *L. jensenii gap1*⁻ for 1 h prior to infection with *N. gonorrhoeae* strain MS11-911 (Em^R). Both *L. jensenii* WT and *L. jensenii gap1*⁻ were able to inhibit gonococcal adherence to epithelial cells; however, the magnitude of inhibition differed

significantly ($p = 0.027$, Figure 4.5). *L. jensenii* WT reduced gonococcal adherence to 59.1 ± 7.0% of the no *Lactobacillus* control ($p = 0.002$, Figure 4.5), similar to previous reports (Spurbeck 2008; Spurbeck 2010). However, *L. jensenii gap1*$^-$ only inhibited gonococcal adherence to 82.0 ± 4.4% of the control ($p = 0.008$ compared to no *Lactobacillus* control (Figure 4.5). These results suggest that *L. jensenii* inhibits gonococcal adherence to epithelial cells using the surface associated GAP1, but this factor is not solely responsible for the inhibition. Adherence of lactobacilli was also measured in these experiments, and the adherence of *L. jensenii gap1*$^-$ (0.57 ± 0.18%), was not significantly different from *L. jensenii* WT (0.40 ± 0.14%, $p = 0.229$). This suggests that although GAP1 is involved in the inhibition of gonococcal adherence, it does not contribute significantly to the adherence of the lactobacilli to the epithelial cells.

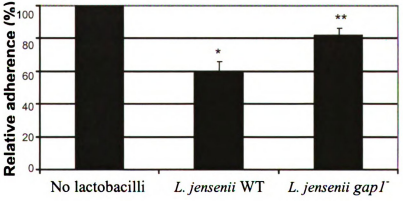

Figure 4.5. Inhibition of *N. gonorrhoeae* adherence to epithelial cells by *L. jensenii* WT and *L. jensenii gap1*$^-$. Relative adherence is the percentage of adherent gonococci in the presence of lactobacilli divided by the percentage of adherent gonococci in the no *Lactobacillus* control. The data are the averages of 3 independent experiments performed in triplicate. * Significantly different from no lactobacilli, ** Significantly different from L. jensenii WT and no lactobacilli. Error bars depict standard error.

Effect of GAP1$^-$ RSC on gonococcal adherence to Hec-1-B cells. Since the GAP1 was first discovered as a part of the RSC that could inhibit gonococcal adherence (155), RSC was prepared from *L. jensenii gap1*$^-$ (*GAP1*$^-$ RSC). Varying concentrations of GAP1$^-$ RSC were tested for inhibitory activity against gonococcal adherence to Hec-1-B cells. GAP1$^-$ RSC reduced the adherence of *N. gonorrhoeae* to epithelial cells in a dose dependent manner, similar to what was observed for RSC from *L. jensenii* WT (155). However, 30% more protein was necessary to reach the level of inhibition seen by RSC preparations derived from wild type (Figure 4.6). These data support the hypothesis that while GAP1 is an inhibitory component of the RSC, there is at least one other inhibitory component present in the released surface components of *L. jensenii* 25258.

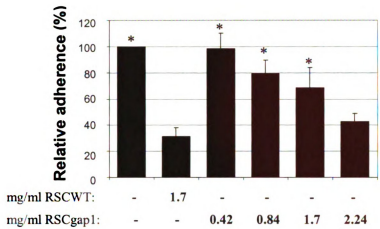

Figure 4.6. Inhibition of gonococcal adherence by released surface components (RSC) from *L. jensenii gap1*$^-$ and *L. jensenii* WT. Gonococcal adherence frequencies are reported as relative to a no RSC control, and *p*-values are reported as compared to WT RSC. Gonococcal adherence to epithelial cells was reduced to 31.2 ± 6.8% (1.7 mg/ml WT RSC), 98.3 ± 12.0% (0.42 mg/ml GAP1$^-$ RSC, $p = 0.004$), 79.1 ± 10.5% (0.83 mg/ml GAP1$^-$ RSC, $p = 0.011$), 68.5 ± 15.4% (1.7 mg/ml GAP1$^-$ RSC, $p = 0.048$), and 42.8 ± 6.2% (2.2 mg/ml GAP1$^-$ RSC, $p = 0.206$) of the untreated control. The data are the averages of 3 independent experiments performed in duplicate. Error bars depict standard error.

Discussion

The primary role of the enzyme enolase is the conversion of 2-phosphoglycerate into phosphoenolpyruvate in glycolysis (16). This reaction is essential to the survival of cells, as this is a crucial step in the production of ATP. However, several cells, including Gram-positive bacteria, eukaryotic parasites, and skeletal myoblasts express enolase on the cell surface, and utilize enolase as a receptor for various substrates (7, 26, 37, 66, 90, 112). Surface-associated enolases from pathogens, parasites, and myoblasts have been shown to enhance the activation of host-derived plasminogen to the proteolytic enzyme plasmin, which then degrades the extracellular matrix of the host (90, 124). This allows pathogens and parasites to invade deep into the host tissues. Myoblasts use this degrading enzyme during tissue repair. However, why lactobacilli have these surface associated enolases is not as clear. Unlike invasive pathogens, which are known to utilize these proteolytic enzymes, lactobacilli do not invade the host, but usually reside in the mucosal layer of either the intestine or the vaginal tract (189). Several *Lactobacillus* species have been found to have surface associated enolases that bind to extracellular matrix proteins such as collagen, fibronectin, and laminin (7, 26, 144); thus it has been proposed that these commensal bacteria utilize surface-associated enolases to colonize the host.

One difference between Gram-positive pathogens and lactobacilli is the number of enolase genes in the genome. Gram-positive pathogens have only one enolase that is present both in the cytosol and on the cell surface, whereas most lactobacilli tested have at least two enolase genes (7, 26). Here we have shown *L. jensenii* ATCC 25258 has two enolase genes in its genome, *gap1* and *eno2*. GAP1 is a

surface associated enolase, while Eno2 is a cytosolic enolase. Other closely related lactobacilli, *L. gasseri* ATCC 33323 and *L. johnsonii* NCC533 have two enolases with high sequence identity to those found in *L. jensenii*, however the cellular localization of the enzymes has not been studied. *L. plantarum*, also has two surface-associated enolases. The enzyme with the highest identity to GAP1 binds fibronectin, which allows *L. plantarum* to colonize colonic epithelial cells *in vitro* (26). Therefore, it appears that several *Lactobacillus* species have evolved to have two proteins to perform separate functions, one surface-associated which is involved in interactions with the host, and the second a cytosolic enzyme functioning in glycolysis.

While GAP1 catalyzes the reaction of 2PGA to PEP *in vitro*, it is likely that it is inactive on the bacterial cell surface *in vivo*, as an inhibitor of enolase activity, Ca^{2+}, is present in the vaginal fluid at 1-5 mM/kg fluid, whereas Mg^{2+}, the cofactor necessary for catalytic activity, has not been detected (181). In fact, the formula for vaginal fluid simulant includes $Ca(OH)_2$ (0.222 g/l) and no magnesium, again demonstrating that GAP1 is most likely inactive in the vaginal tract (121). Furthermore, in the cell culture medium (DMEM, Invitrogen) used to initially identify GAP1 as an inhibitor of gonococcal adherence to epithelial cells, there is 2.26-fold more calcium than magnesium, which would render the enzyme inactive in the cell culture assays. This supports the finding that the enzymatic activity is not important for inhibition of gonococcal adherence in this *in vitro* system (Figure 3). In addition, we demonstrated that the substrate-binding site is necessary for the inhibition of gonococcal adherence, suggesting that the structure of GAP1 is essential for the inhibitory activity. Therefore, we propose that although this protein has retained the

ability to act as an enolase, that it is not functioning as an enolase when present on the surface of *L. jensenii*.

Bacteria for alternative functions have adapted several enzymes known to be active in central metabolism. These "moonlighting enzymes", while retaining significant homology to known enzymes, can often act as receptors on the cell surface. In this group of moonlighting proteins are several glycolytic enzymes, including fructose-1,6-bisphosphate aldolase (168), enolase (8, 26), phosphoglycerate kinase (24) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, (8, 78, 165)). All of these enzymes have been shown to mediate pathogen interactions with the host, either through adherence to host cells (7-8, 26, 78, 168), evasion of the immune system (165), or by blocking the invasion of host tissues (24). Therefore, surface-associated glycolytic enzymes may play a central role in bacterial-host interactions, and deserve further study.

We propose that *L. jensenii* produces a surface-associated enolase, GAP1, to reduce competition with other bacteria in the vaginal tract. Other lactobacillar enolases are known to bind to extracellular matrix proteins such as collagen, fibronectin, and laminin (7, 26, 144), and we have previously shown the inhibition of gonococcal adherence by released surface components from *L. jensenii* can be removed by incubation with soluble fibronectin (155). Therefore, it is plausible that an interaction between GAP1 and host cell receptors or extracellular matrix proteins, such as fibronectin, reduces gonococcal adherence to epithelial cells. The receptors gonococci adhere to on the host cells are bound by the active site of GAP1, thus reducing the available substrate for gonococci to colonize.

Treatment and prevention of STIs, such as gonorrhea, is a significant public health challenge, particularly considering the rapid development of antibiotic resistance. The discovery of GAP1 from *L. jensenii* could lead to new therapeutic breakthroughs for one of the most common infectious diseases in the United States.

CHAPTER FIVE

SIGNIFICANCE AND IMPACT OF THIS WORK

Vaginal infections are a common problem for women worldwide. Several infections, including vulvovaginal candidiasis and bacterial vaginosis, can develop into recurrent infections, even in women who are treated with antimicrobial compounds. The ineffectiveness of these treatments necessitates the search for alternative treatments or prevention methods to treat these persistent infections. Women can also be re-infected with *N. gonorrhoeae*, one of the most common sexually transmitted pathogens in the United States. This disease is treatable with antibiotics; however, the infectious agent, *Neisseria gonorrhoeae*, is now classified as a multidrug resistant "superbug", and thus is becoming more difficult to treat. To date, the CDC only recommends one class of antibiotics to be used to treat gonorrhea, third generation cephalosporins (www.cdc.gov). The high incidence of the disease, coupled with the bacterium's increasing resistance to antibiotics, produces a need to study potential alternative methods to treat or prevent gonorrhea.

One potential alternative for the treatment or prevention of vaginally acquired infections is the use of probiotics, defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit upon the host". Another alternative treatment or preventative could be products derived from these beneficial microbes. Ideally, probiotics and their products should be safe for continuous use in order to reduce the risk of recurrent infections. One of the most studied genera for use as probiotics is *Lactobacillus*. Lactobacilli are thought to protect the vaginal tract against incoming pathogens, including STIs such as gonorrhea. However, before this

work, there was no experimental evidence that lactobacilli could inhibit gonococcal interactions with the host epithelial cells.

In this dissertation *Lactobacillus jensenii*, one of the four main species found in the healthy human vaginal tract, was shown to inhibit gonococcal adherence and invasion of epithelial cells in culture (Chapter 2, (154)). The process of adherence and invasion are the initial steps in the gonococcal infection: if the bacteria cannot adhere to the epithelial cells in the endocervix, the pathogen cannot produce a sustainable infection. Therefore, the ability of *L. jensenii* to inhibit these steps implies that this organism, or its products, may have potential to be used as a method for reducing the transmission of gonorrhea. However, *L. jensenii* was unable to significantly displace gonococci already adherent to epithelial cells, which suggests that these microbes are limited to the prevention of gonorrhea. I have found that another species of *Lactobacillus*, *L. gasseri* ATCC 33323, was able to displace adherent gonococci and, therefore, does have potential to provide an alternative treatment. However, *L. gasseri* only displaced ~30% of the adherent gonococci, and may need to be genetically modified to increase the displacement for effective treatment to be achieved (Chapter 2, (154)).

The mechanism(s) probiotic organisms utilize to prevent pathogen adherence is an area that has not been thoroughly pursued, hampering the development of probiotics as treatments or prophylactics. Therefore, I sought to determine the molecular mechanism(s) of the inhibition of gonococcal adherence to epithelial cells by *L. jensenii* (Figure 5.1).

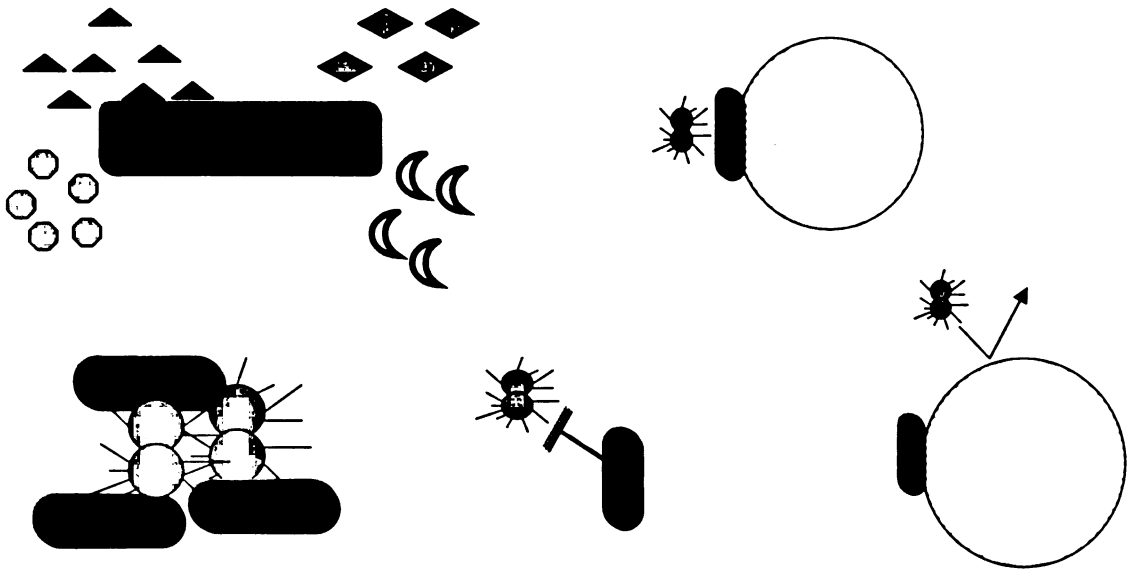

Figure 5.1. Potential probiotic mechanisms of inhibition of pathogen adherence. A. Secreted compounds such as (◆) lactic acid, (☾) hydrogen peroxide, (▲) biosurfactants, or (●) bacteriocins. B. Direct competition for host cell receptors. C. Co-aggregation with the pathogen. D. Inhibition of virulence gene expression. E. Inducing the host epithelial cells to become more resistant to infection.

After eliminating several proposed mechanisms of inhibition, including direct killing, co-aggregation, and secreted inhibitory factors, I determined that *L. jensenii* inhibits gonococcal adherence using at least two surface associated protein components, one of which is an enolase (Chapter 3). This enolase, GAP1, is a glycolytic enzyme that converts 2-phosphoglycerate to the higher energy compound phoshoenolpyruvate (16). This enzyme inhibits gonococcal adherence by utilizing the substrate-binding site, but the dehydratase activity is not required for this inhibition (Chapter 4). Therefore, I propose a model by which *L. jensenii* inhibits gonococcal adherence by actively depositing GAP1 on the surface of the epithelial cells or on the extracellular matrix. This enolase binds to an as of yet unknown epithelial cell receptor, thereby inhibiting the adherence of *N. gonorrhoeae* to the same receptor, or by preventing gonococci from reaching a different receptor in close proximity to where GAP1 binds (Figure

5.2). One potential receptor for GAP1 is fibronectin, since the addition of soluble fibronectin to the RSC abolished the inhibition of gonococcal adherence (Chapter 3).

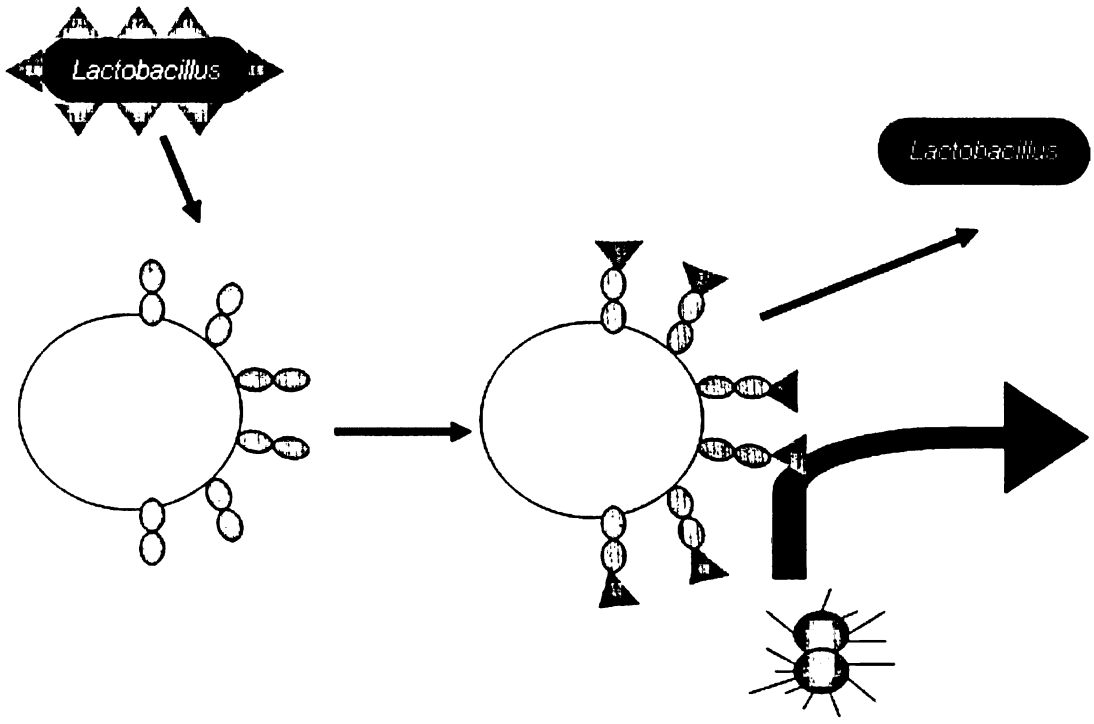

Figure 5.2. Proposed mechanism by which *L. jensenii* GAP1 inhibits gonococcal adherence. *L. jensenii* deposits its surface bound GAP1 on epithelial cell receptors. This blocks the adherence of *N. gonorrhoeae* to the same or neighboring receptors.

The discovery of a protein produced by *L. jensenii* that can inhibit gonococcal adherence is significant, in that this protein should be safe for use as a prophylactic against gonorrhea. Further studies need to be conducted to determine the efficacy of preventing other vaginal infections, as well as how effective this protein is *in vivo*. Once it is known whether *L. jensenii* or GAP1 can be used to prevent gonorrhea, or other vaginal infections, the organism or its product could be marketed and used to reduce the incidence of gonorrhea. This would greatly relieve the burden on our health care system incurred by the treatment of these diseases and their sequelae.

My work demonstrates that the conventional way pathogenesis research is conducted, where researchers study pathogen interactions with the host in isolation, needs to be broadened to include the indigenous microbiota to yield realistic results. Rarely is it the case that a pathogen solely interacts with a site of the host not already colonized by commensals. As shown in this work, bacteria that are indigenous to the human body can drastically affect the interactions of a pathogen with the host. In the field of probiotics, researchers need to pay particular attention to the mechanism by which the beneficial microorganisms inhibit pathogen interactions with the host. By understanding how a probiotic works, better predictions can be made about the efficacy of treatment for different diseases.

FUTURE EXPERIMENTS

While this work has made significant contributions to understanding how *L. jensenii* effects gonococcal interactions with the host epithelial cells, there are still many unanswered questions that need to be addressed in future experiments. First, fluorescently labeled antibodies specific to the GAP1 can be used to confirm that the GAP1 from *L. jensenii* does bind the host epithelial cells by visualizing the bound protein through confocal microscopy. Next, the epithelial cell receptor potentially blocked by the binding of GAP1 should be determined. Gonococci are known to bind several host cell receptors, however, the cell line used to examine the effects of *L. jensenii* on gonococcal adherence, Hec-1-B, only express a subset. On Hec-1-B cells, gonococci bind to extracellular matrix proteins (34, 41, 174), glycolipids (126), heparin sulfate proteoglycans (50, 109), lutropin receptors (153), and CD46 (69). Taking into consideration the substrate specificity of the active site of the GAP1,

which is involved in the inhibition of gonococcal adherence to Hec-1-B cells, the ligand found on the cell surface should be negatively charged, or contain a phosphate group and/or a carboxyl group (16). To determine the cell receptor, one could utilize antibodies to each receptor for gonococcal adhesins to try to block the GAP1 from binding. This could be visualized by confocal microscopy, and then the affinity to each receptor could be quantified by an ELISA and western blot. An alternative method would be to allow the GAP1 to bind to Hec-1-B cell lysates and cross-link the protein with bound ligand. A western blot could be used to identify proteins bound by the GAP1. Such proteins can then be excised from an SDS-PAGE gel and sent for tandem mass spectrometry to determine their molecular characteristics and biological properties.

Once the ligand is determined on the epithelial cells, the residues necessary for binding should be examined. The active site of the GAP1 is located on the C terminal end of the TIM β-barrel domain, therefore, the specific amino acids necessary for binding the substrate will be found in the $\beta\alpha$ loops on this end, as well as the C terminal ends of the β sheets (186). To determine which residues are necessary for the inhibitory action against gonococcal adherence, site-directed mutagenesis should be performed to systematically substitute the charged polar amino acids in these regions with nonpolar amino acids. By aligning the GAP1 sequence from *L. jensenii* 25258 with several surface associated and intracellular enolases, 19 candidate residues have been identified (Figure 5.3).

```
1   MSVITDIHAR EVLDSRGNPT AEAEVYTELG GFGRAIVPSG ASTGEHEAVE LRDGDKSRFG 60
    *  * #  *# *  ******* ^*^# ###^* ^ *  ^**** ***** **** ****## *@#

61  GQGVLTAVEN VNGEIAKAVI GLDVTDQRLI DQTMIDLDGT PNKGRLGANA ILSVSLASAR 120
    *^**^^**#* ** @*#^  #     @#*^ * *^@**@**** **** ***** ** ** *^*

121 AAADELGLPL YEYLGGPNAH VLPTPMMNVI NGGKHADNNV DIQEFMIMPV GAKSLHEAVR 180
    ***#@   ** *^****# ^^   * # #*   ***^* *      ******#** ##^  ^   #

181 MGAETFHTLK GLLQERGEST AVGDEGGFAP NLKNNEEPFE ILVEAIQRAG YKPGQDIAIA 240
    ## #^#* *    * @#*^^*   ******** # @  *   #   # #*^#** * **^

241 FDCAASEFYN KDTKKYVTVA D--GREYTAEEW TSLIEDLVDK YPVISVEDPL DENDWEGWKT 300
    #* *@*#**@   ^ @* ^ ^ ^^^*@^^*  *  ^^    *  # #* *  **@# ****  * #

301 FTERLGDKVQ IVGDDLFVTN TSYLEKGIKM GVANSILIKL NQIGTLTETF EAIEMAKEAG 360
     *# ##*  *  *#**  #** # ^*^ #*@^ *  *##***  ***** ***   #*# #  #*

361 YTAVVSHRSG ETEDTTIADL VVATNAGQIK TGSMSRTDRI AKYNQLMRIE EALGSTAQYK 420
    * #  ***** **#* #* *   **#*#** * **# #*  *  #***#* #**  ^*#^ #^#

421 GIHSFYNLHK QF 432
    # ^#####^  ^^
```

Figure 5.3. The peptide sequence of *L. jensenii* GAP1. ^ only in *L. jensenii* like group, # in *L. jensenii* like group and pathogen group, @ only in *Lactobacillus* groups, and * conserved in all groups. In bold are residues of interest for site directed mutagenesis. The gray shaded region corresponds to the TIM barrel domain.

These amino acids are found in the closely related enolase sequences from *L. jensenii* 25258, *L. gasseri* 33323, and *L. johnsonii*, and therefore, these could potentially be involved in modifying the substrate specificity to include the eukaryotic ligand. A cytoplasmic enolase could also be tested for the ability to inhibit gonococcal adherence to epithelial cells. If the cytoplasmic enolase does not inhibit gonococcal adherence, this protein could undergo site directed mutagenesis to change specific residues to those found in GAP1 from *L. jensenii* 25258 to see which are sufficient for inhibition of gonococcal adherence. If the cytoplasmic enolase is found to inhibit gonococcal adherence, then its sequence should be compared with that of GAP1 to determine if there is a signal sequence or other motif that determines the cellular localization of each enzyme.

One region of interest is the last 14 residues at the C-terminus. This could be a potential localization sequence, as this sequence is highly conserved between most surface-associated enolases, or may be necessary for the binding of an epithelial cell receptor (figure 5.3). In *Streptococcus pyogenes*, this region of a surface bound enolase was found to contain two lysine residues necessary for the binding of plasminogen (35). To determine if the 14 amino acids of the C-terminus are necessary for localization or inhibition of gonococcal adherence, a deletion of this region could be made and then expressed in *L. jensenii gap1*$^-$ on a plasmid. Enolase activity of whole cells could then be compared between the insertional knockout and the deletion mutant. If the deletion mutant can complement the knockout, then the 14 amino acids of the C-terminus is not involved in localization to the cell surface. If, however, the deletion mutant does not complement the surface associated activity, then this region should be further analyzed by a series of truncations or single amino acid substitutions to determine the consensus sequence that is necessary and sufficient for cell surface localization. The deletion mutant should also be examined for the ability to inhibit gonococcal adherence, since the result would shed light on the role of this region in that phenomenon.

L. jensenii was found not only to inhibit *N. gonorrhoeae* adherence to epithelial cells, but to inhibit gonococcal invasion of the host cells as well. However, the effect of either the RSC or GAP1 from *L. jensenii* on gonococcal invasion has not been examined. Therefore, invasion assays should be performed to determine if GAP1 or other inhibitory components found in the RSC mediate the inhibition of gonococcal invasion. The prevention of gonococcal invasion of host epithelial cells could reduce

the severity of the disease; therefore knowing what lactobacillar component mediates this inhibitory effect would be beneficial for the development of new therapeutics.

Finally, identification of the other inhibitory protein(s) present in the RSC should be completed. One candidate is the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) present in the first inhibitory fraction. Although this enzyme is normally thought to be cytosolic, GAPDH has been found on the cell surface of other lactobacilli as well as pathogenic bacteria (8, 35, 78). Furthermore, GAPDH has been shown to mediate the binding of some *Lactobacillus* species to colonic mucin, and extracellular matrix proteins such as fibronectin (78, 144). To determine if GAPDH is involved, anion exchange chromatography followed by size exclusion chromatography could be conducted to further isolate the protein from the RSC. Then the protein could be assayed for inhibitory activity against gonococcal adherence. The gene for GAPDH in *L. jensenii* should also be identified and cloned into an expression vector to obtain purified His_6-tagged protein. The purified enzyme could then be tested for inhibition of gonococcal adherence to epithelial cells. Once the other inhibitory protein(s) present in the RSC is determined, the mechanism of inhibition can then be examined. Once this research has been completed, therapeutics may be designed to treat or prevent gonorrhea and possibly other vaginal infections, thus improving women's health in our society.

APPENDIX

Spurbeck, R. R. and Arvidson, C. G. (2010). Subtractive hybridization method to isolate organism-specific RNA from a mixed bacterial sample.

ABSTRACT

Microarray analysis is an extremely powerful technique to examine whole transcriptome changes in response to different conditions. Quantitative PCR is typically used to corroborate or expand upon microarray data. These techniques require purified RNA, which limits their application to single organism systems. Since we are interested in the response of *Neisseria gonorrhoeae* to the vaginal microbiota, we developed a subtractive hybridization technique to obtain highly purified gonococcal RNA from samples also containing *Lactobacillus jensenii*.

INTRODUCTION

Microarray analysis of gene expression is a widely used method to examine global changes in gene expression in response to various experimental conditions, and is a powerful tool for the study of bacterial pathogenesis. Approaches to the study of gene expression in pathogens that involve analysis of transcript levels are powerful because changes in response to environmental cues can indicate potential therapeutic targets. Most studies of bacterial pathogenesis utilize an idealized system containing only the pathogen in question and host cells. However, *in vivo*, pathogens are in a complex environment containing the indigenous microbiota with which they often interact (83). Our laboratory studies the sexually-transmitted bacterial pathogen, *Neisseria gonorrhoeae*. We have developed and used a DNA microarray to examine global gene expression in gonococci in a tissue culture model of infection (38-39) and have also begun to examine the effects of commensal lactobacilli on gonococcal interactions with host cells (154-155). In order to overcome the problem of isolating gonococcal RNA from samples containing lactobacilli, it was necessary for us to develop a technique to subtract *L. jensenii* RNA out of a mixed sample, leaving behind purified RNA of *N. gonorrhoeae* that can be used for gene expression studies.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Neisseria gonorrhoeae* MS11 [P^+ Tr (148)] was grown on GC agar (Accumedia, Lansing, MI) with supplements (75) and VCNT inhibitor (Becton, Dickinson, and Company, Sparks, MD) at 37°C with 5% CO_2 (+ CO_2) in a humidified atmosphere. *L. jensenii* [ATCC 25258, $H_2O_2^+$ (156)], was grown on MRS agar at 37°C + CO_2 in a humidified atmosphere (Difco, Sparks, MD).

Co-culture conditions and RNA isolation. Lactobacilli and gonococci were swabbed from overnight plate cultures into Dulbecco's modified Eagle medium without phenol red (DMEM, Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (Invitrogen), 110 mM sodium pyruvate, and GC supplement II [12.4 μM $Fe(NO_3)_3$]. Bacterial concentrations were determined spectrophotometrically (A_{600}). In a 13 x 100 mm cell culture plate, 4 ml of DMEM was inoculated with 10^9 CFU/ml *L. jensenii* and incubated for 1 h at 37°C + CO_2. The culture was then inoculated with 10^9 CFU/ml *N. gonorrhoeae* and incubated 3 h at 37°C + CO_2. The bacterial suspension was then transferred to a tube containing an equal volume of ice cold MeOH and incubated on ice for 5 min before centrifugation to harvest the bacteria. Controls in which only *N. gonorrhoeae* or *L. jensenii* were present in the culture were treated similarly. The bacteria were resuspended in 200 μl lysosyme (10 mg/ml in H_2O) to lyse the gonococci. RNA was isolated from each sample using the Qiagen RNeasy kit according to the manufacturer's protocol (Qiagen, Valencia, CA) and the concentration was quantified spectrophotmetrically (A_{260}).

Genomic DNA isolation. *L. jensenii* was grown overnight in 10 ml MRS broth, with shaking at 37°C. The bacteria were harvested by centrifugation and then resuspended in 1 ml STE buffer (6.7% sucrose, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 0.25 U/μl mutanolysin (Sigma-Aldrich, St. Louis, MO), and incubated for 30 min at 37°C. 300 μl of 20% SDS was added, and the sample was mixed and incubated at 42°C for 30 min. DNA was then extracted by phenol:chloroform:isoamyl alcohol (25:24:1) three times. The sample was treated with RNase, and then extracted with chloroform. DNA was then ethanol precipitated, resuspended in TE buffer, and quantified spectrophotmetrically (A_{260}).

Subtractive hybridization. To remove *L. jensenii* RNA from RNA samples, an equal amount of *L. jensenii* genomic DNA was added to each RNA sample, and brought to 30 μl with TE. The sample was gently mixed by inversion, and incubated at 42°C for 25 min. The RNA:DNA mixture was chilled on ice and 5 μl of RNase H (10 U/μl, Ambion Inc., Austin, TX) was added. The sample was then incubated at 37°C for 45 min, followed by 5 min incubation at 65°C to inactivate the enzyme. The sample was then treated with DNase I (Ambion) according to the manufacturer's directions to remove the remaining *L. jensenii* DNA from the sample.

The RNA samples were then reverse transcribed using SuperScript II Reverse Transcriptase according to the manufacturer's directions (Invitrogen). The concentration of cDNA was determined spectrophotmetrically (A_{260}) and then diluted to 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, and 1 pg/μl. The cDNA was then amplified by semi-quantitative PCR (30 cycles) using primers specific to the 16S rDNA gene of *L. jensenii* and *pilT* from *N. gonorrhoeae*. Since this was not an end point PCR,

differences in the amount of product can be visualized and correspond to the amount of template present.

RESULTS AND DISCUSSION

To date, very few researchers have attempted gene expression studies of co-cultured organisms (83), limiting the scope of these experiments to organisms which can be lysed differentially (101). Since we are interested in the response of *N. gonorrhoeae* to the host environment, which includes indigenous lactobacilli, we have added to the differential lysis approach a subtractive hybridization step to remove the *Lactobacillus* RNA from a mixed sample leaving only pure gonococcal RNA for transcription studies.

Due to the differences in the cell walls of *L. jensenii*, a Gram-positive organism, and *N. gonorrhoeae*, a fragile Gram-negative organism, samples containing gonococci and lactobacilli were gently lysed with lysozyme (10 mg/ml), which efficiently breaks open the gonococci leaving the lactobacilli largely intact. The lysate was centrifuged to remove unbroken lactobacilli and RNA purified from the supernatant using a Qiagen RNeasy kit. However, this purified RNA was found to contain a significant amount of *Lactobacillus* RNA, presumably due to a small amount of lysis in the original sample. To remove the *Lactobacillus* RNA the mixed RNA sample was incubated with an excess of genomic DNA from *L. jensenii* allowing RNA:DNA hybrids to form. The sample was then treated with RNase H, which selectively degrades RNA:DNA hybrids by specifically hydrolyzing the phosphodiester bonds of the RNA hybridized to the DNA (163). A subsequent DNase

I step was done to remove the remaining *L. jensenii* DNA, leaving only pure RNA from *N. gonorrhoeae*.

To validate this method, we utilized semi-quantitative, reverse transcription PCR (semiQ RT-PCR) to determine the amounts of *L. jensenii* and *N. gonorrhoeae* RNA present before and after treatments. As seen in Figure A.1, row B, the RNase H treatment completely removed the *L. jensenii* RNA, leaving no template cDNA for PCR amplification. The *N. gonorrhoeae* RNA remained after treatment as demonstrated by semiQ RT-PCR of the *pilT* gene (Figure A.1, row A). These results demonstrate that this method can remove unwanted bacterial RNA from a mixed sample to undetectable levels, yet leaving other RNA in the sample intact. Applications for this subtractive hybridization method would be to purify bacterial RNA for microarray or real-time RT PCR analysis of transcriptional changes in one microbe grown in the presence of another microbe (or microbes), such as pathogens grown in the presence of organisms of the commensal microbiota.

CONCLUSIONS

A method of subtractive hybridization has been developed to remove the RNA of one microorganism from a mixed sample leaving purified RNA from a single bacterial species. The technique of hybridizing the commensal RNA with genomic DNA from the same species, followed by selective degradation of the RNA:DNA hybrid is effective and specific. This will allow us to expand the applicability of techniques for analyzing gene expression to include research into bacterial interactions.

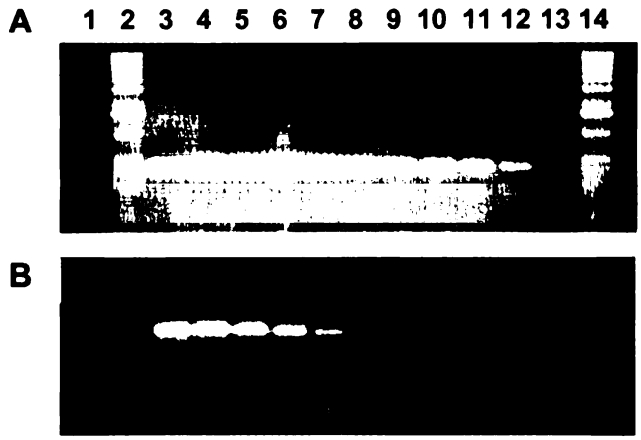

Figure A.1. Semi-quantitative PCR of *L. jensenii* 16S rDNA (panel B) and *N. gonorrhoeae pilT* (panel A). A. Lane 1: no template control; Lane 2: 1 kb ladder; Lane 3: *N. gonorrhoeae* genomic DNA; Lanes 4-8: 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, and 1 pg/μl cDNA from *N. gonorrhoeae*; Lanes 9-13: 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, and 1 pg/μl cDNA *N. gonorrhoeae*-mixed with *L. jensenii* which was subjected to the subtractive hybridization method to remove the *L. jensenii* RNA before reverse transcription; Lane 14: 1 kb ladder. B. Lane 1: no template control; Lane 2: 1 kb ladder; Lane 3: *L. jensenii* genomic DNA; Lanes 4-8: 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, and 1 pg/μl cDNA from *L. jensenii*; Lanes 9-13: 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, and 1 pg/μl cDNA *N. gonorrhoeae*-mixed with *L. jensenii* which was subjected to the subtractive hybridization method to remove the *L. jensenii* RNA before reverse transcription, and Lane 14: 1 kb ladder.

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